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The reductive dechlorination of chlorinated phenols by vitamin B₁₂, supported by the reductant Ti(III) citrate was examined. Procedures were developed, including a novel reactor system, for conducting these experiments. Most of the experiments were conducted in either hermetically-sealed glass ampoules, which could maintain vitamin B₁₂ in the fully-reduced vitamin B_{12s} state for months, or in the novel two-chambered reactor (TCR), which could also support vitamin B_{12s} for extended periods, and could be easily sampled to perform kinetic studies.

Vitamin B_{12s} reductively dechlorinates chlorinated phenols by nucleophilic aromatic substitution, followed by reductive cleavage of the aryl cobalamin to form the reductively-dechlorinated product. Dechlorination occurs chiefly at positions *ortho* to another chlorine, but the 2 and 6 positions are extremely recalcitrant, even when another chlorine is adjacent. The proposed mechanism accounts for the observed regiospecificity, consistent with bond charge density and thermodynamic considerations.

Pentachlorophenol, all of the tetrachlorophenols, and all of the trichlorophenols were challenged with vitamin B_{12s}, and all were reductively dechlorinated to some extent. A sequential dechlorination pathway was constructed and compared to patterns exhibited by anaerobic microbial consortia. On the basis of this comparison, it was concluded that any involvement of vitamin B₁₂ in biological reductive dechlorination of chlorinated phenols requires the intimate participation of apoenzymes to direct the regiospecificity toward the 2 and 6 chlorines on the ring.

The observed kinetics of the reductive dechlorination of pentachlorophenol by vitamin B_{12s} can be described with a first order approximation. A threefold variability in rate constants was observed. The rate of reductive dechlorination of pentachlorophenol by vitamin B_{12s} is insufficient to account for reductive dechlorination by acclimated bacteria.

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The Ph.D. degree has been my dream for many years now. I have pursued this goal at three institutions on opposite sides of the country, and through it all I have enjoyed the loving attention of my wife, Debby. She has always supported my ambitions, wherever they have taken me. I am truly blessed.

I dedicate this thesis to my parents, Frank and Janie Smith, who instilled in me the love of learning, and who have always stood by me as I continued my studies. They have rejoiced in my triumphs and offered solace in my disappointments, and had the wisdom to let me rise and fall on my own, but never fall too hard. As I travel and meet new people in new places, I am increasingly grateful for what they provided that I once thought was commonplace, but now realize is all too rare— a happy home.

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Reductive Dechlorination of Chlorophenols by Vitamin B₁₂

Chapter 1

Introduction

Of the many environmental pollutants that pose industrial waste processing and environmental remediation challenges, many are halogenated hydrocarbons. By halogenating a molecule, a new chemical can be designed with unique chemical and physical properties suitable for a specific application. Chlorine is the most common halogen used.

The carbon-chlorine bond is quite stable and resistant to oxidation, properties which are in part responsible for their usefulness. Chlorinated hydrocarbons are, however, slowly biodegraded both aerobically and anaerobically (Perry, 1979; Vogel and McCarty, 1985; Vogel et al., 1987; Tiedje et al., 1987; Mohn and Tiedje, 1992). Under the appropriate conditions, the chlorine can be released and the carbon to which it is attached reduced. This process is, therefore, known as reductive dechlorination. Many metalloorganic compounds, including biological cofactors such as hematin, vitamin B₁₂, and coenzyme F₄₃₀, can also abiotically catalyze reductive dechlorination. Reductive dechlorination by these cofactors has been proposed as an abiotic model of the anaerobic biodegradation of chlorinated hydrocarbons (Schrauzer and Katz, 1978; Krone et al., 1989a,b; Gantzer and Wackett, 1991).

Recently, vitamin B₁₂ was reported to reductively dechlorinate pentachlorophenol, with Ti(III) citrate as the source of electrons (Gantzer and Wackett, 1991). The products of this reductive dechlorination were the result of removal of chlorines *meta* and *para* to the hydroxyl group. This regiospecificity is in contrast to that of unacclimated anaerobic microbial consortia, which preferentially reduce the position

ortho to the hydroxyl, but resembles the reductive dechlorination of pentachlorophenol by certain acclimated consortia (see below). The reductive dechlorination of aliphatic chlorides provides no comparable opportunity to investigate the mechanism of chemical action through analysis of products.

The research described herein arose from attempts to repeat Gantzer and Wackett's (1991) pentachlorophenol reductive dechlorination using vitamin B₁₂ as catalyst and dithiothreitol as reductant. Little, if any, dechlorination was detected, with some results suggesting that *ortho* dechlorination might be occurring. I had believed, from reading the original paper, that dithiothreitol had been successfully used to support pentachlorophenol reductive dechlorination. A careful rereading of their paper revealed that only Ti(III) citrate was used as a reductant for their pentachlorophenol experiments. When the reductant was changed to Ti(III) citrate, relatively rapid dechlorination at the *meta* and *para* positions was observed. I also noticed that dithiothreitol-reduced vitamin B₁₂ was yellow, while Ti(III) citrate-reduced vitamin B₁₂ was blue. A literature review revealed that the yellow color is indicative of the Co(II) oxidation state, vitamin B_{12r}, while the blue color indicates the completely reduced Co(I) state, vitamin B_{12s}. The intriguing possibility that two different oxidation states of vitamin B₁₂ could both reductively dechlorinate pentachlorophenol, and with different regiospecificity, suggested that the dechlorination pattern could be directed by the reducing potential of the medium, and might in part explain the phenomenon of acclimation.

Goals and Objectives

The overall goal of this research was to develop an increased understanding of the role of reducing potential and metal coenzymes in reductive dechlorination. Preliminary experiments had suggested an experimental system which would explore

the role of vitamin B₁₂ in the reductive dechlorination of pentachlorophenol. This experimental system was attractive for several reasons.

First, vitamin B₁₂ and related corrinoids are present in high concentrations in methanogenic and acetogenic bacteria (Dangel et al., 1987; Zeikus et al., 1985; Wolf, 1985; Krautler et al., 1987, 1988; Stupperich et al., 1988). They and related organometallic cofactors have been implicated in alkyl halide transformations by biological systems, and their effectiveness as catalysts of reductive dechlorination of alkyl and alkenyl halides has been recently reported (Krone et al., 1989a, b; Gantzer and Wackett, 1991).

Second, the chemistry of vitamin B₁₂ has been extensively studied for over 40 years and was (I believed) well understood. Vitamin B₁₂ is readily available from any of several chemical supply houses, relatively inexpensive, and active in the proposed system. It was, therefore, a reasonable catalyst to study.

Third, pentachlorophenol had only recently been reported to be reductively dechlorinated by vitamin B₁₂, and offered virgin territory for scientific investigation. If vitamin B₁₂ further reductively dechlorinated lesser chlorinated chlorophenol congeners, the regiospecificity might suggest a mechanism.

Fourth, pentachlorophenol and its congeners are common environmental pollutants, and study of their degradation is quite practical. Numerous studies of the biological reductive dechlorination of pentachlorophenol and its congeners have collectively established dechlorination pathways that indicated that the *ortho* chlorine is most readily replaced by unacclimated anaerobic consortia. The *meta* and *para* chlorines are labile only to acclimated anaerobic consortia. Reductive dechlorination of pentachlorophenol and its congeners by vitamin B₁₂ could therefore be of interest not only *en bloc*, but in comparison to biological reductive dechlorination, and might

suggest environmental manipulation of waste treatment or remediation schemes to facilitate the mineralization of chlorophenols or other chlorinated hydrocarbons.

Fifth, catalytic reductive dechlorination as a practical treatment or remediation scheme would require development of appropriate catalysts, conditions, and modeling parameters. This research would provide the basis for further development of such schemes.

In pursuit of the research goal, the following objectives were established:

1. Determine the dechlorination pathways of the model catalytic system, using Ti(III) citrate as the source of electrons.
2. Obtain kinetic data from the model catalytic system and use the data to model the progress of the reductive dechlorination of pentachlorophenol.
3. Compare the kinetics and pathways of vitamin B₁₂-catalyzed reductive dechlorination to those for biological processes.
4. Determine the effect of reducing potential, i.e., the valence state of the vitamin B₁₂, on kinetics and pathways.

Dissertation Overview

A review the chemical and physical properties of chlorinated hydrocarbons, including their known reactions relative to the present work, is presented in Chapter two. Chapter three reviews the chemistry of vitamin B₁₂ relative to its role as a catalyst of reductive dechlorination. Chapter four reviews the biological reductive dechlorination of chlorinated hydrocarbons, with special emphasis given to the chlorophenols.

Chapters five through eight were written in a form suitable for publication individually as journal articles or research notes. Chapter five describes three

techniques for maintaining extreme reducing conditions for extended periods of time which were developed in the course of this study and have proven useful. Chapter six describes the pathways for the sequential dechlorination of pentachlorophenol and all of the tetra- and trichlorophenols, and proposes a mechanism for vitamin B₁₂ catalysis. Chapter seven describes the kinetics of pentachlorophenol reductive dechlorination and further develops the proposed mechanism in relation to the observed kinetics. Chapter eight describes the results of attempted reductive dechlorination of pentachlorophenol by vitamin B₁₂, reduced either by dithiothreitol or by limited amounts of Ti(III) citrate.

Chapter nine summarizes the research and suggests areas for further inquiry. Chapter ten summarizes the conclusions made possible by this research. The appendices provide detailed descriptions of the procedures used in this research.

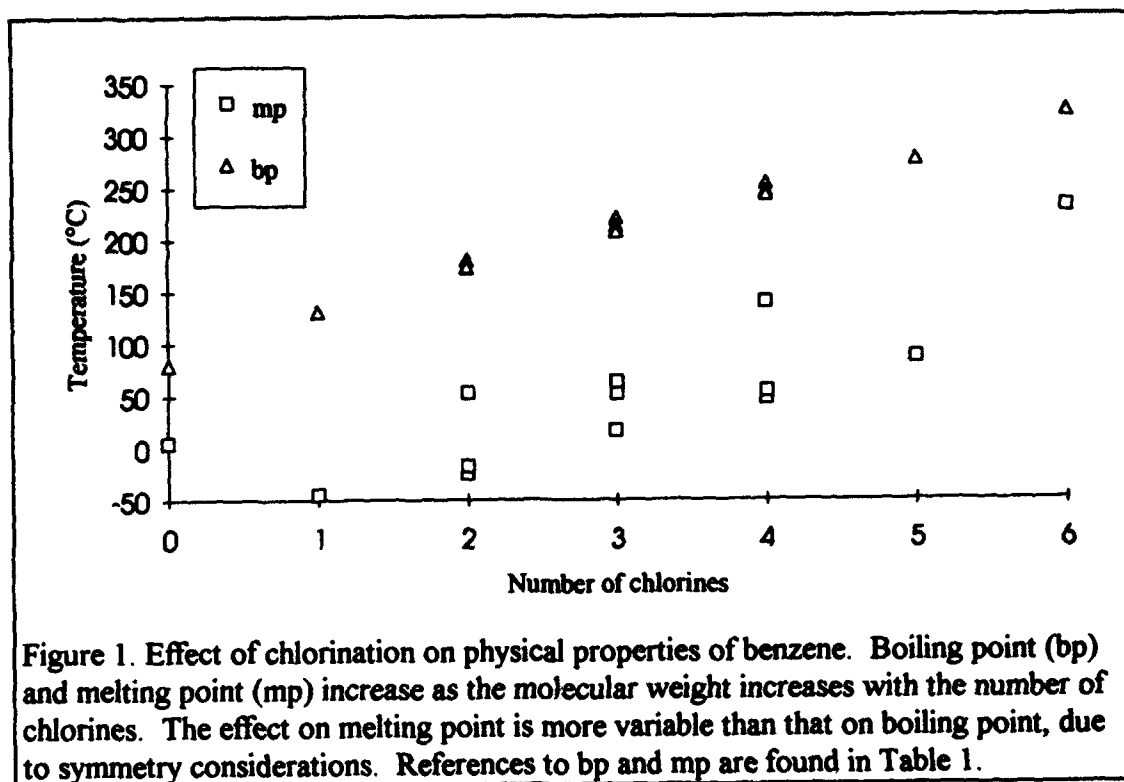
Chapter 2

Physical and Chemical Properties of Halogenated Hydrocarbons

Halogenation of organic chemicals is an important method of creating compounds with unique properties. Chlorine is by far the most common halogen in commercial products. In general, adding a chlorine will raise the boiling point, by increasing the molecular weight. The melting point is not affected in such an orderly fashion, due to the relatively more complex process of crystallization as compared to vaporization, but the overall trend is the same (Table 1; Figures 1 and 3). Note the significantly higher melting point of *para*-dichlorobenzene over the other dichloro isomers, and the higher melting point of 1,2,4,5-tetrachlorobenzene over the other tetrachloro compounds. These two compounds are much more symmetrical than the other isomers, which significantly reduces the orientational energy barrier to crystallization. By the same token, the melting point of chlorobenzene is actually lower than benzene. The sixfold rotational axis and two of three twofold rotational axes are lost by chlorination, making chlorobenzene a much less stable solid.

Chlorination will alter the polarity of the molecule when the relatively electropositive hydrogen is replaced by the relatively electronegative chlorine. Sometimes polarity is increased (compare dichloromethane, highly polar, to methane, nonpolar), and sometimes decreased (compare chloroform, polar, to carbon tetrachloride, nonpolar). This greatly affects the solubility and solvent properties of the substance. Sequential chlorination of alkanes or alkenes produces compounds with increasing boiling points, and with solvent properties reflecting the changing polarity. The highly electronegative chlorine can also affect the activity of other functional groups. Adding chlorines to phenol increases the acidity of the phenolic group (Table 1, Figure 2) by withdrawing electrons and stabilizing the phenolate anion.

Table 1. Effect of chlorination on the physical properties of chlorobenzenes and chlorophenols.			
Compound	mp	bp	pK
Benzene	5.5	80.1	
Chlorobenzene	-45	131	
<i>m</i> -Dichlorobenzene	-24.76	173	
<i>o</i> -Dichlorobenzene	-17.03	180.5	
<i>p</i> -Dichlorobenzene	53.5	174.1	
1,2,3-Trichlorobenzene	52.6	221	
1,2,4-Trichlorobenzene	17	213	
1,3,5-Trichlorobenzene	63.4	208.4	
1,2,3,4-Tetrachlorobenzene	47.5	254	
1,2,3,5-Tetrachlorobenzene	54.5	246	
1,2,4,5-Tetrachlorobenzene	139.5	243	
Pentachlorobenzene	86	277	
Hexachlorobenzene	231	323	
phenol	42	180	10.0
<i>o</i> -chlorophenol	7	175	8.56
<i>m</i> -chlorophenol	31	216	9.12
<i>p</i> -chlorophenol	43	217	9.41
2,3-dichlorophenol	58	206	7.70
2,4-dichlorophenol	43	210	7.89
2,5-dichlorophenol	58	210	7.51
2,6-dichlorophenol	66	220	7.79
3,4-dichlorophenol	67	253	8.63
3,5-dichlorophenol	68	233	8.19
2,3,4-trichlorophenol	81	subl.	7.66
2,4,5-trichlorophenol	65	246	7.43
2,4,6-trichlorophenol	68	246	6.23
2,3,5-trichlorophenol	62	249	7.37
2,3,6-trichlorophenol	58	272	5.96
3,4,5-trichlorophenol	101	275	7.84
2,3,4,5-tetrachlorophenol	117	subl.	6.6
2,3,4,6-tetrachlorophenol	67		5.45
2,3,5,6-tetrachlorophenol	114	subl	5.48
pentachlorophenol	190	309	4.5
Boiling points and melting points (°C) of chlorophenols, Renner (1990). pK's, Dolfing and Harrison (1992). All other data, Weast (1970).			



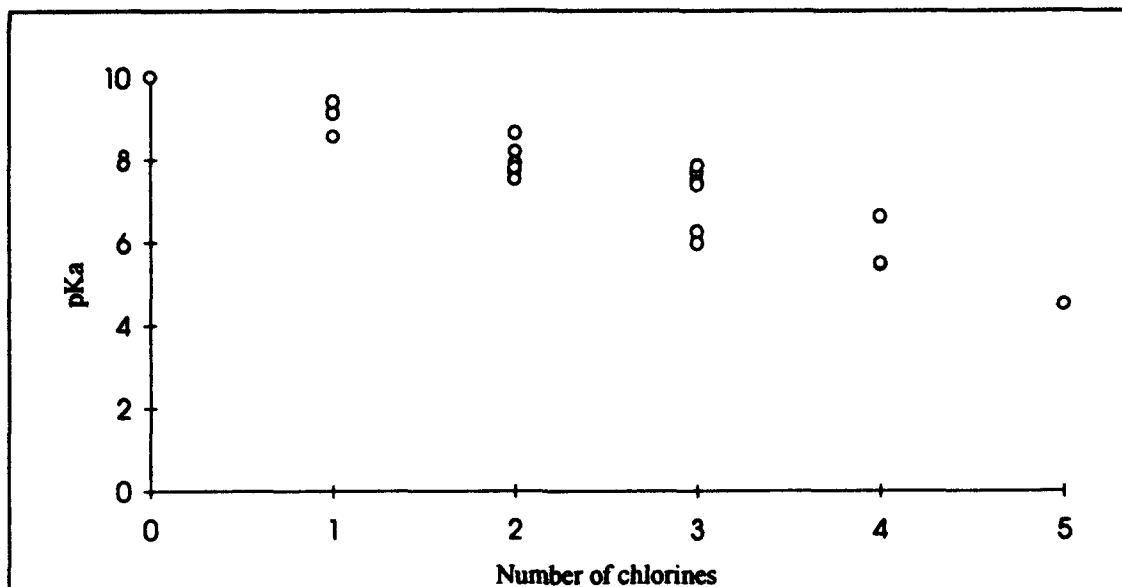


Figure 2. Effect of chlorination on ionization of the phenolic group. The electron-withdrawing chlorine atoms stabilize the phenolate anion, decreasing the pK (increasing the acid character) as the degree of chlorination increases. Data references are found in Table 1.

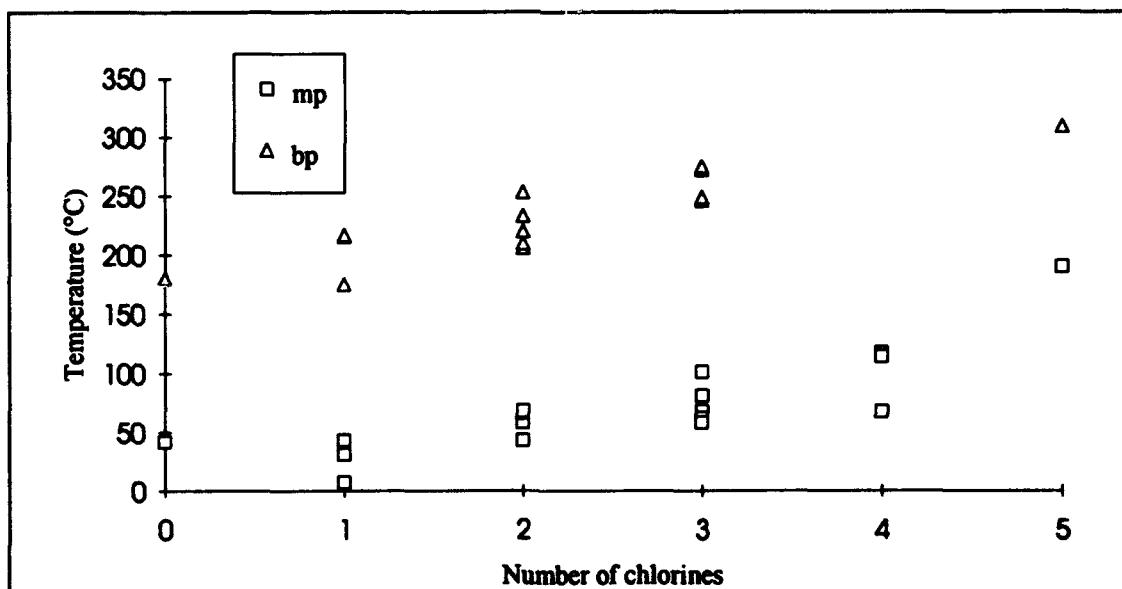


Figure 3. Effect of chlorination on the physical properties of phenol. Boiling point and melting point increase as the molecular weight increases with the number of chlorines. Data references are found in Table 1.

Halogens can be displaced from organic halides by several mechanisms. The carbon-halogen bond is highly polarized, the chlorine being relatively electron rich, while the carbon is relatively electron poor. The carbon is therefore susceptible to attack by a nucleophile. The nucleophile replaces the chlorine in a process known as nucleophilic substitution. Nucleophilic substitution of aliphatic halides and aromatic halides occur by quite different and distinct mechanisms. Unless otherwise noted, the following discussion of reaction mechanisms is condensed from McMurray, 1992.

Nucleophilic Substitution of Alkyl Halides

In one type of nucleophilic substitution in aliphatic halides, the kinetics are first order in the nucleophile and first order in the organic halide, or second order overall. The entire process is known as nucleophilic substitution, bimolecular, abbreviated S_N2 . A key feature of this reaction is the inversion of the substituents on the reacting carbon (Figure 4). The attacking nucleophile approaches the reacting carbon from directly behind the departing chlorine. There is a transition state with the carbon in a pentacoordinate configuration. This transition state is unstable, so that the reaction essentially proceeds in one step from reactants to products. Since the halide leaves as an anion, the more stable the anion, the more readily does an S_N2 reaction occur. The reactivity of the halides as a leaving group is in the order $I^- > Br^- > Cl^- > F^-$.

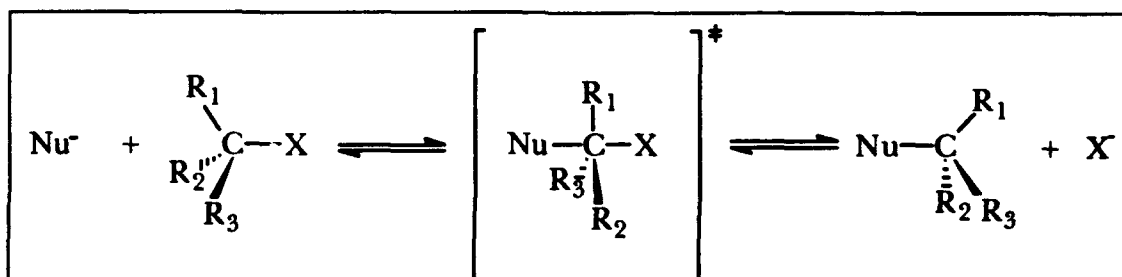
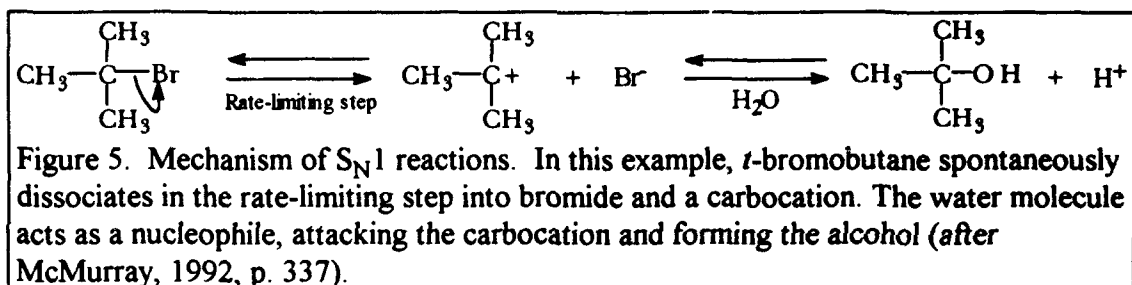


Figure 4. Mechanism of S_N2 reactions. The nucleophile approaches from the opposite side of the halide, forming an unstable pentacoordinate carbon species which breaks down to the substituted compound and halide ion. The process shown occurs in one step. (Figure adapted from McMurray, 1992, p. 365.)

Nucleophilic substitution of alkyl halides can also occur by a unimolecular (first order) process, abbreviated S_N1 . In this reaction, the halide spontaneously dissociates from the rest of the molecule, leaving a carbocation, which reacts with the nucleophile to form the substitution product (Figure 5). The stereochemistry about the reacting carbon is not necessarily inverted, but optically active substrates are partially racemized. Inversion is favored, perhaps because the halide that has just left partially blocks nucleophilic attack at that position. S_N1 reactions are favored by conditions which stabilize the carbocation intermediate. Tertiary halides are most reactive. The order of reactivity is $3^\circ > 2^\circ \approx \text{benzyl} \approx \text{allyl} > 1^\circ > \text{methyl}$.



Leaving groups affect reactivity the same as for the S_N2 reaction, because the leaving group leaves as an anion in this case as well. Since the rate-limiting step in an S_N1 reaction is the spontaneous dissociation of the organic halide into a halide ion and a carbocation, the nature of the nucleophile has no bearing on the rate of an S_N1 reaction.

Alkyl halides can also undergo dehydrohalogenation when there is a β -hydrogen available (Figure 6). In the bimolecular elimination ($E2$) reaction, a strong base attacks the β -hydrogen. The halide simultaneously begins to depart and a carbon-carbon double bond develops. Unimolecular elimination can also occur, where the halide leaves spontaneously, leaving a carbocation. Subsequent nucleophilic attack of the base on the β -hydrogen yields the alkene in a fast (non-rate-limiting) reaction. The $E1$

and S_N1 reactions often compete, yielding products which are mixtures of substitution and dehydrohalogenation.

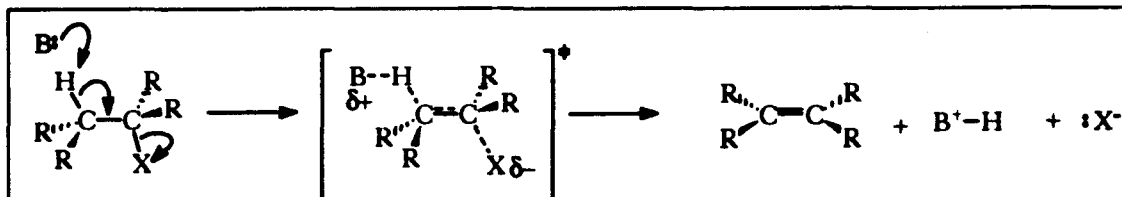


Figure 6. Dehydrohalogenation of alkyl halides by the E2 mechanism. As a strong base ($B:$) attacks the β hydrogen, the halide starts to leave and the carbon-carbon double bond starts to form. The completed reaction leaves a halide and alkene. (Figure adapted from McMurray, 1992, p 389).

Nucleophilic Substitution of Aryl Halides

Aryl halides are not subject to nucleophilic attack by the S_N2 mechanism, because the side of the carbon opposite to the halide is blocked by the ring, and inversion of the carbon is impossible. Neither are aryl halides subject to S_N1 reactions under normal circumstances, because the aryl cation is very unstable.

Aryl halides are subject to nucleophilic substitution by different mechanisms. When, in addition to the halide, there are electron-withdrawing groups *ortho* or *para* to the halide, the aryl halide can react with nucleophiles by nucleophilic aromatic substitution (S_NAr). The mechanism of S_NAr has two distinct steps, the addition of the nucleophile followed by the elimination of the halide. The nucleophile attacks the relatively electron-deficient carbon, forming a negatively-charged intermediate called a Meisenheimer complex (Figure 7). Unlike the pentacoordinate carbon transition state in the S_N2 reaction, the Meisenheimer intermediate is fairly stable, containing a tetravalent carbon σ -bonded to the attacking nucleophile. The intermediate is also sometimes called a σ -complex. The σ -complex is stabilized by electron-withdrawing groups *ortho* and *para* to the site of attack; stabilization of the σ -complex will

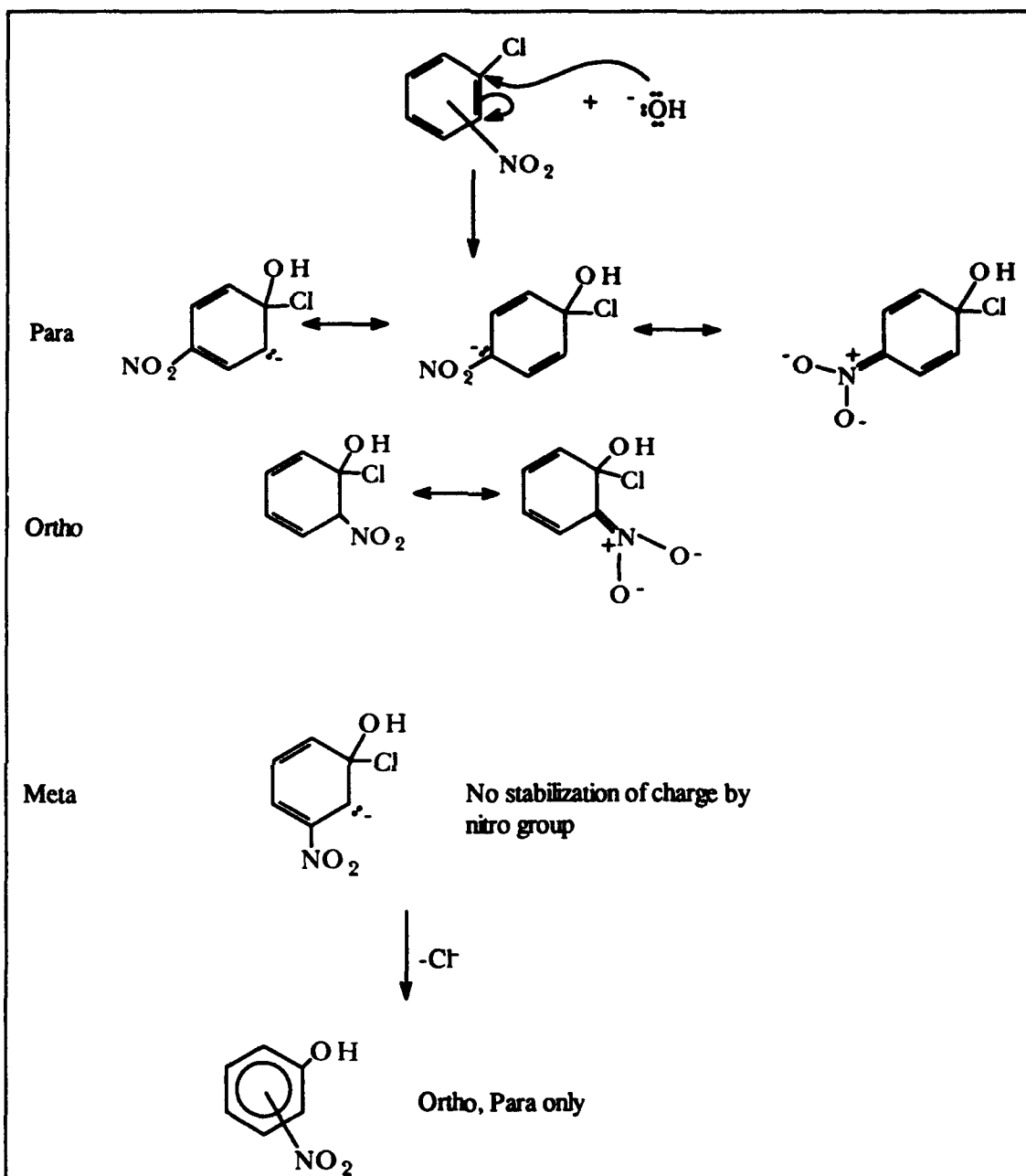
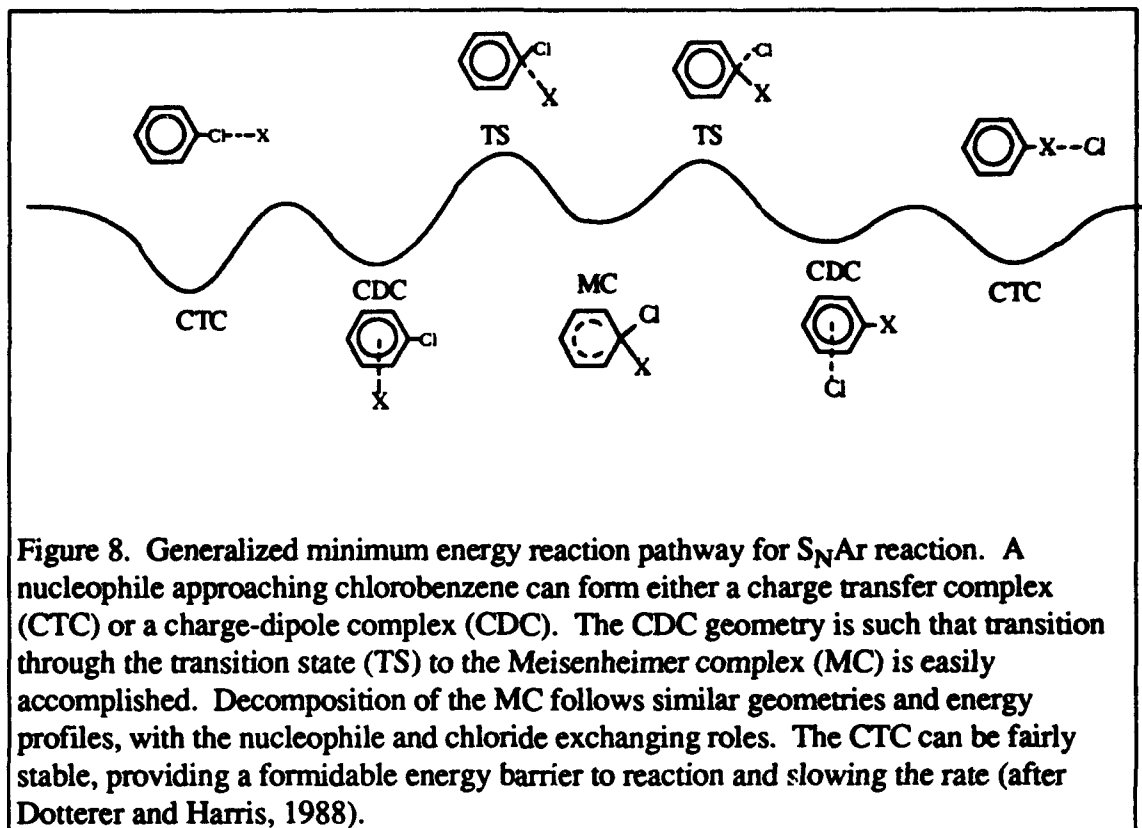


Figure 7. Addition-elimination (S_NAr) reaction mechanism. A nucleophile, in this case hydroxide ion, attacks the aryl halide at the relatively electron-deficient halogenated carbon, forming a σ -bond. The intermediate is known as a Meisenheimer complex or σ -complex, and is resonance stabilized by electron-withdrawing groups *ortho* and *para* to the site of substitution. (Figure adapted from McMurray, 1992, pp. 589-590.)

promote the reaction. Aryl halides with no further substitution, or those substituted *meta* to the halide even with strongly electron-withdrawing groups, are unreactive under normal circumstances. The vast majority of the literature on σ -complexes discuss activation by nitro groups. Halides are usually the groups displaced. It is generally accepted that it is the electron withdrawing capacity of the nitro groups that is responsible for the stabilization of the σ -complex, and that this generalization would apply to other electron-withdrawing groups (McMurray, 1992).

In addition to the stable Meisenheimer intermediate, two other energy minima exist along the reaction coordinate¹ (Dotterer and Harris, 1988). If the nucleophile approaches the halide head on, collinear to the halogen-carbon bond, there is partial charge transfer from the nucleophile anion to the arene (Figure 8). This complex has been termed a charge transfer complex (CTC). A similar complex, the charge-dipole complex (CDC), more closely resembles the geometry of the Meisenheimer complex. In the CDC, the nucleophile lies above the ring at about the Van der Waals distance, but there is very little charge transfer and its energy minimum is shallow compared to the CTC. A nucleophile-aryl halide interaction that forms the CTC must move out of this linear configuration into the side-by-side configuration in order to proceed to the Meisenheimer complex. Should a nucleophile-arene pair find itself in an especially stable CTC, the progression of the reaction will be hindered.

¹Based on modified neglect of differential overlap (MNDO) calculations.



When electron-withdrawing groups are arranged about a benzene ring in a 1,3,5 pattern, even a protonated carbon can be activated towards nucleophilic attack and formation of the σ -complex (Servis, 1965, 1967). Diethylamine and 1,3,5-trinitrobenzene react in DMSO according to the scheme in Figure 9. Trinitroanisole treated with sodium methoxide in methanol first forms the σ -complex at the 3 position (3σ), which then resolves to the more familiar and thermodynamically-favored 1σ -complex (Figure 10).

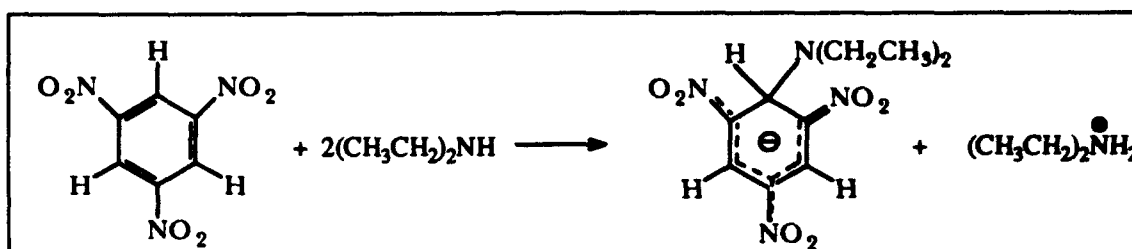


Figure 9. Reaction of 1,3,5-trinitrobenzene with diethylamine in DMSO to form the σ -complex. (Figure redrawn from Servis, 1967.)

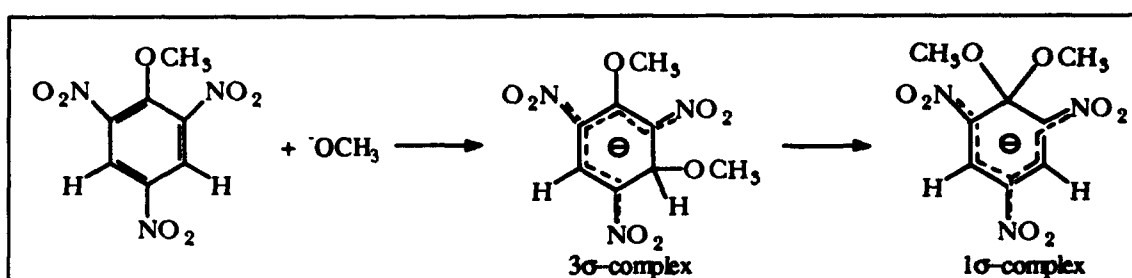


Figure 10. Nucleophilic addition of trinitroanisole by methoxide ion in methanol solution. Nucleophilic attack results first in the kinetically-favored 3σ -complex, which decays to the thermodynamically more stable 1σ -complex. (Figure adapted from Servis, 1967.)

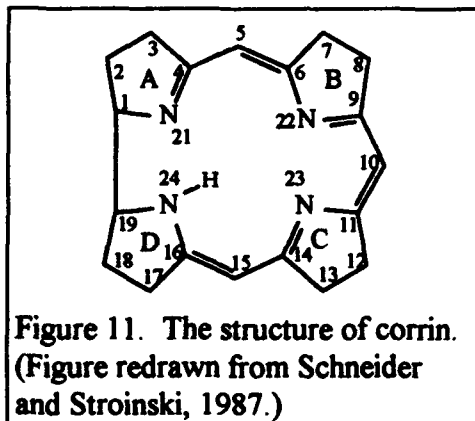
Chapter 3

The Chemistry of Vitamin B₁₂

Reviewing the chemistry of vitamin B₁₂ is complicated by the diversity of the literature and the complexity of the molecule itself. Studies have been published by organic chemists, inorganic chemists, biochemists, electrochemists, and physical chemists, and the perspective of each specialty differs greatly. Often, these authors describe similar phenomena with different terminology, and the unwary reader may not recognize it as such. What is alkylation of cobalt to an inorganic chemist is nucleophilic substitution to an organic chemist, and these two specialists will describe this identical process quite differently.

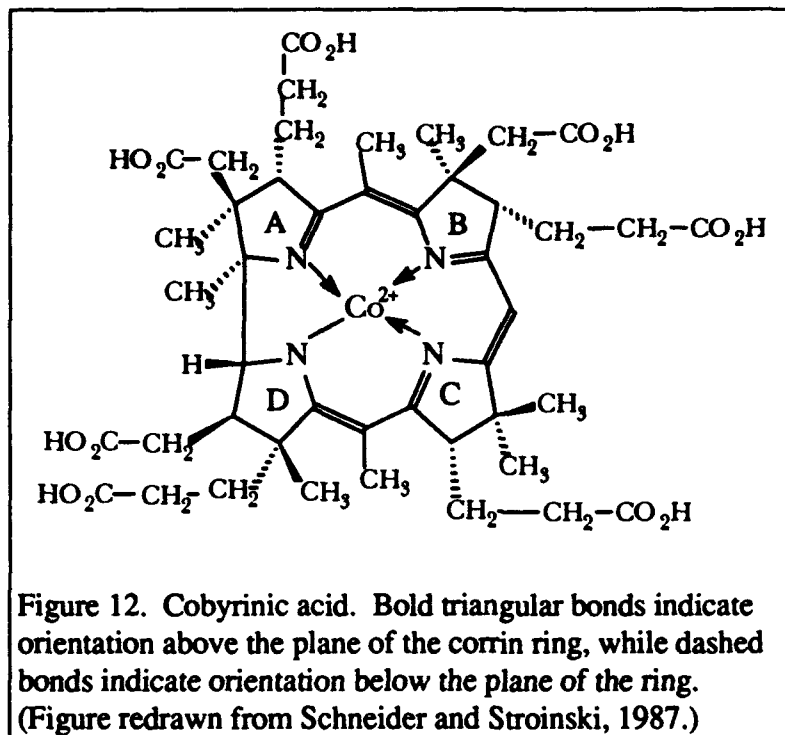
Nomenclature

The following is a condensation of the 1973 recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature (Schneider and Stroinski, 1987; Cohn, 1982) and is intended to acquaint the reader with nomenclature necessary to understand the ensuing discussion. For further details the reader should consult the referenced works.



Vitamin B₁₂ is an organometallic compound consisting of a cobalt atom coordinated at four positions by the nitrogens of a corrin ring (Figure 11), a structure similar to the perhaps more familiar porphyrin found in heme. Corrin differs from porphyrin in the linkage between rings A and D. Carbons 1 and 19 are joined directly in corrin, while in

porphyrin a methylene carbon intervenes. Corrin numbering follows that of porphyrin, position 20 being omitted to preserve the similarity. All **corrinoids**, including vitamin B₁₂, share the common characteristic of a corrin ring.



Many natural corrinoids contain cobalt and a common array of substituents on the corrin nucleus.

Cobyirinic acid

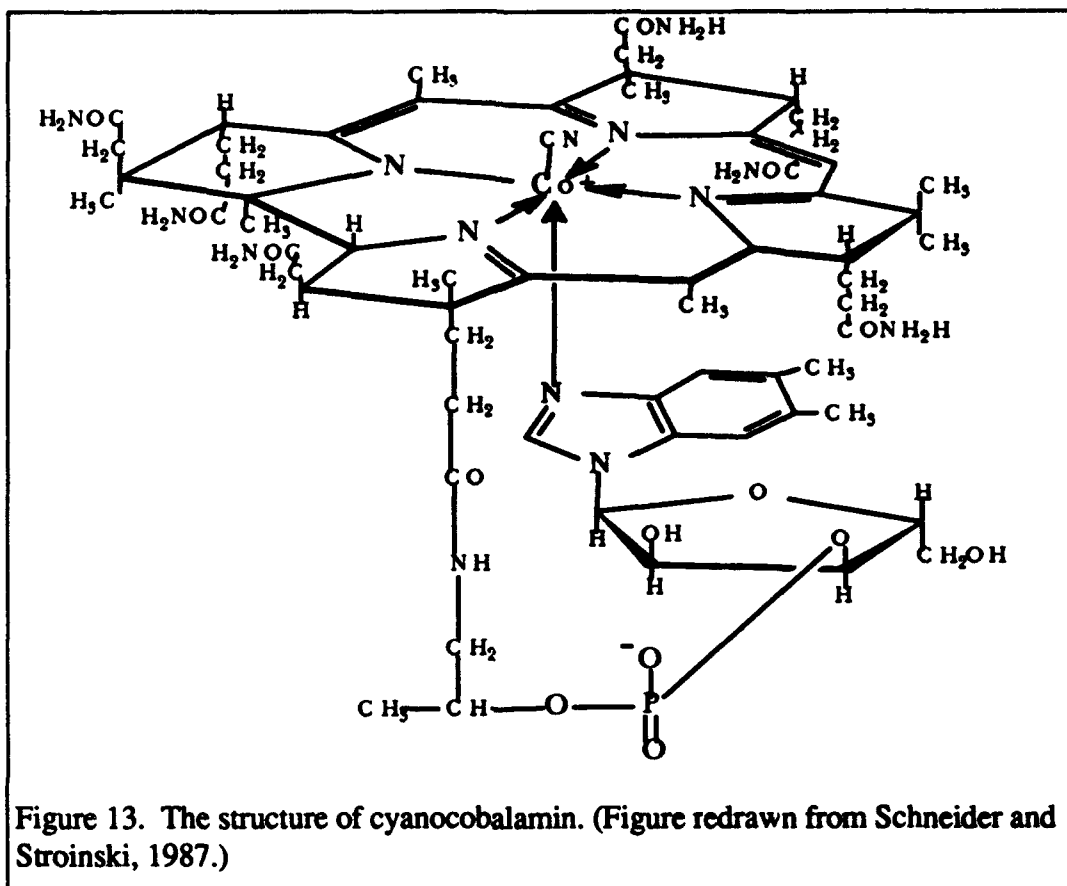
(Figure 12) contains methyl groups and seven two- or three-carbon carboxylate groups attached to the ring. **Cobinamide**

consists of cobyirinic

acid, of which six of the carboxylic acids are converted to their corresponding amides, the seventh carboxyl being joined by an amide linkage to D-1-amino-2-propanol.

Cobamide is cobinamide with an α -D-ribofuranose 3-phosphate residue joined to the 2 position of the aminopropanol. Further substitution of 5,6-dimethylbenzimidazole by a glycosyl linkage to the ribose leads ultimately to vitamin B₁₂, or **cobalamin** (Figure 13). The latter is represented in chemical use by the short symbol Cbl.

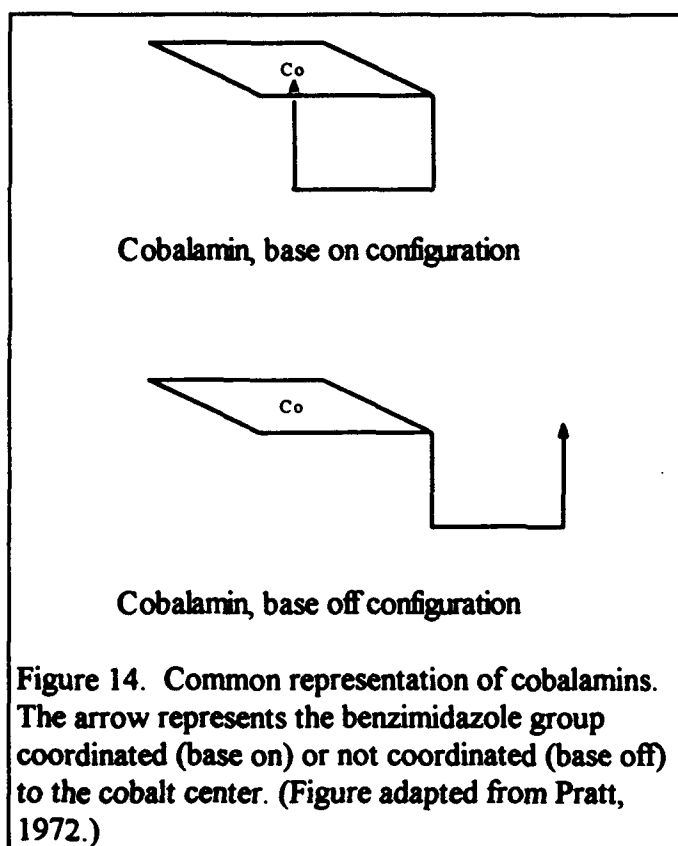
Cobalt, at the center of the planar corrin, can exist in three oxidation states, +1, +2, or +3. Depending on the oxidation state, the cobalt will coordinate with additional ligands. These additional ligands will lie necessarily above or below the plane of the corrin ring, as on the axis of a top, and are referred to as **axial ligands**. Trivalent cobalt



is preferentially hexacoordinate. Two axial ligands lie one at each axial position. One of these ligands is usually a nitrogen of the benzimidazole group described above. This position of attachment is defined as the cobalt- α position, and is usually drawn in diagrammatic representation below the corrin plane. This configuration is assumed to exist unless otherwise indicated. The sixth ligand is assumed to be above the plane, in the cobalt- β position, without further reference. Thus, cyanocobalamin (Figure 13) is a cobalamin with cobalt in the +3 oxidation state, the benzimidazole group coordinated to the cobalt at the α position and a cyanide ion at the β position. The β ligand usually is relatively loosely bound and can be fairly easily replaced. However, much of the unique chemistry of vitamin B₁₂ is due to the fact that the β position can be occupied by a covalently bound carbon. Vitamin B₁₂ coenzyme contains methyl or adenosyl

groups at the β position (methylcobalamin or adenosyl cobalamin), and is considered to be the metabolically active form of the molecule.

Divalent cobalt is preferentially pentacoordinate, a condition usually satisfied by the four corrin and one benzimidazole nitrogens. Monovalent cobalt is preferentially tetracoordinate, which is satisfied by the corrin nitrogens alone. The preferred orientations do not preclude additional ligands, but additional ligands would be bound less firmly.



The corrin ring is often represented by a parallelogram drawn to depict the plane of the corrin viewed along the edge, with the Co drawn in the center (Figure 14). In this representation, a ligand below the plane is considered to be in the α position. Above the plane is the β position. A bent line below the plane, sometimes with an arrow head, denotes the benzimidazole group. The end of the line pointed toward the

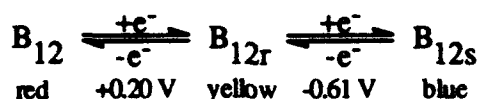
cobalt denotes the **base on** configuration; pointed downward or away from the cobalt denotes a **base off** orientation.

Reactions of Vitamin B₁₂

The reactions of vitamin B₁₂ are summarized in Figure 15. The form of vitamin B₁₂ commercially available is cyanocobalamin, a name synonymous with the simple trivial name vitamin B₁₂. The cyanide may be replaced by water to make aquo-cob(III)alamin, also known as vitamin B_{12a}. Either of these can be reduced by the stepwise addition of electrons into cob(II)alamin (vitamin B_{12r}) and cob(I)alamin (vitamin B_{12s}). The standard reduction potentials for each step are +.20 V (vs. SHE) for aquocob(III)alamin → cob(II)alamin and -0.61 V for cob(II)alamin → cob(I)alamin (Lexa and Saveant, 1983). At high pH, the water ligand is ionized to OH⁻, which, being a stronger ligand, inhibits the first reduction. When sufficient potential is finally reached to reduce the cobalamin to cob(II)alamin, the next reduction proceeds apace. At pH 12, a two-electron reduction is observed directly from vitamin B_{12a} to vitamin B_{12s} at about -0.66 V (Lexa and Saveant, 1976; de Tacconi et al., 1979).

Cob(II)alamin behaves chemically much like a free radical, and can be alkylated by alkyl radicals (reaction 2a, Figure 15). Cob(II)alamin can abstract a halogen from an alkyl halide, leaving an alkyl radical to react with an additional cob(II)alamin to form the alkylated cobalamin (reaction 2b, Figure 15) (Halpern and Maher, 1965; Blaser and Halpern, 1980). The kinetics for alkyl chlorides and bromides follow the second-order rate expression $-d[B_{12r}]/dt = 2k[B_{12r}][RX]$, interpreted as donation of an electron from vitamin B_{12r} to the halogen and formation of the alkyl free radical, followed by rapid combination of the free radical with a second vitamin B_{12r} to form the alkylated cobalamin. Organic iodides (reaction 2c, Figure 15), react second order in vitamin B_{12r}, third order overall. This was interpreted by a rapid equilibrium between vitamin B_{12r} and the organic iodide, followed in the rate-determining step by the donation of an

1. Electrochemical interconversion (Lexa and Saveant, 1983)

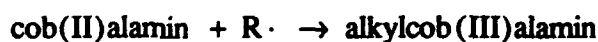


2. Reactions of vitamin B_{12r}

2a. Condensation with free radicals



2b. Reactions with alkyl halides (except iodide) (Halpern and Maher, 1965; Blaser and Halpern, 1980).



Overall:



2c. Reactions with alkyl iodide (Blaser and Halpern, 1980).



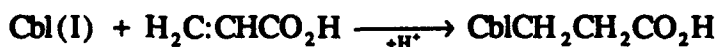
3. Reactions of vitamin B_{12s}

3a. Substitution of organic halides (Johnson et al., 1963; Schrauzer and Deutsch, 1969)



R = alkane, alkene, alkyne (Johnson, et al., 1963)

3b. Addition.



(Johnson, et al., 1963)

Figure 15. Reactions of vitamin B₁₂.

electron by a second vitamin B₁₂. The products are alkylated cobalamin, aquocobalamin, and iodide (Blaser and Halpern, 1980).

Cobalt(I) corrinoids are among the strongest nucleophiles known, due to the spin-paired highest occupied molecular orbital (HOMO) electrons in the dz^2 (axial) orbital (Schrauzer et al., 1965). With this enormous nucleophilicity, vitamin B₁₂ in nature is highly selective for the methyl carbonium ion, which explains the key role of vitamin B₁₂ in single-carbon chemistry. Cobalt(I) corrinoids readily attack organic halides and replace the halide (Figure 15, reaction 3a). For alkyl halides, the substitution follows an S_N2 mechanism (Schrauzer and Deutsch, 1969). Cob(I)alamin can also substitute for halogen in halogenated olefins and alkynes (Johnson et al., 1963).

Olefins and alkynes will also react with cob(I)alamin to result in addition products (Figure 15, reaction 3b) (Johnson et al., 1963). Alkynes, being more electrophilic, react more readily than do alkenes. Ethylene is essentially unreactive, but activating groups (such as carboxyl) on the alkene will promote addition to the carbon β to the activating group. The reaction of halogenated alkynes with cob(I)alamin results in a mixture of the substitution and addition products.

Once alkylated, the cobalamins may be thought of as existing in one of three states (Figure 16), either a cob(I)alamin complexed with a carbocation, cob(II)alamin complexed with a free radical, or cob(III)alamin complexed with a carbanion (Pratt, 1972). Varying of the axial ligand in the series CN⁻, HCC⁻, CH₂CH⁻, CH₃CH₂⁻, the behavior of the complexes changes from that typical of cobalt(III) complexes (hexacoordinate, high formation constant) to that of cobalt(II) (pentacoordinate, low formation constant). There is no clear equilibrium, and it is perhaps easiest to think of them all as the cobalt(III) complex.

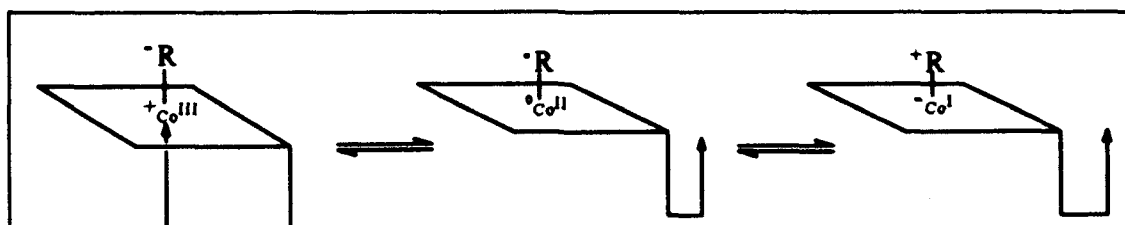


Figure 16. Alternative representations for alkylcobalamins. Depending upon the ligand strength of the alkyl group, the organocobalamin may exist as a Co(III)-carbanion pair, a Co(II)-radical pair, or a Co(I)-carbocation pair. (Adapted from Pratt, 1972.)

Reactions of Organocobalamins

Organocobalamins can undergo several different reactions. Spontaneous homolytic cleavage of the Co-C bond results in cob(II)alamin and organic free radical:

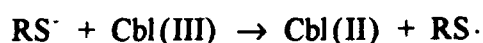


The equilibrium is probably far to the left (Pratt, 1972). Some alkyl cobalamins are among the most stable organometallic compounds known, being stable in the dark up to 200°. The addition of heat or light overcomes the endothermic nature of the reaction; many organocobalamins are quite light labile. The products of the homolytic cleavage can be seen if removed by further reaction. Photolysis of methylcobaloxime in the presence of cob(II)alamin yields methylcobalamin, evidence of the free radical reaction with the cob(II)alamin (Schrauzer et al., 1968). The major product of the photolysis of methylcobalamin is ethane, formed by the condensation of two methyl radicals. When the ligand is bulky, such as a secondary alkyl group, the complex can dissociate simply from the steric strain (Schrauzer and Grate, 1981).

Methylcobalamin will transfer the methyl group to the Lewis acid Hg(II), yielding methyl mercury (Hill, 1970). This reaction may be an important source of organic mercury pollutants (Wood et al., 1968). In electrochemical reduction experiments at controlled potential, a one-electron reduction of methylcobalamin resulted in the

release of methyl radical, which could be either further reduced to methane or scavenged by the mercury from the apparatus electrode to form methyl mercury (Hill, 1971).

A one-electron oxidation by thiol radicals has been proposed to account for the methylation of the thiols by methylcobalamin (Agnes et al., 1971). The reaction requires the presence of catalytic amounts of O_2 to generate the thiol radicals, followed by the following scheme:



Primary alkylcobalamins with bulky groups β to the cobalamin, and secondary cobalamins can spontaneously undergo sterically-induced bond cleavage (Grate and Schrauzer, 1979; Schrauzer and Grate, 1981). If the β carbon of the resulting alkyl radical contains a hydrogen, an alkene can form by elimination of the β hydrogen by concerted elimination, with the hydrogen in a *syn* configuration relative to the cobalamin (Figure 17). These sterically-strained cobalamins are more stable in acid solution, where protonation of the benzimidazole prevents coordination at the cobalt α position, allowing the corrin ring to deform to accommodate the bulky ligand. At neutral or basic pH, the benzimidazole resumes its coordination position. The resulting steric strain forces the bond cleavage.

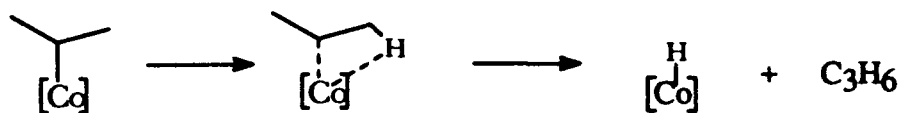


Figure 17. Concerted β elimination of sterically-strained secondary organocobalamin. Steric strain of the bulky secondary alkyl group against the corrin ring induces the formation of the olefin. The cobalamin and the β hydrogen leave in concert (Figure redrawn from Grate and Schrauzer, 1979).

In the absence of a β hydrogen, the sterically-induced cleavage is homolytic, resulting in the organic free radical and cob(II)alamin (Figure 18) (Schrauzer and Grate, 1981). Normally, the two free radicals recombine rapidly. In the presence of oxygen, however, the organic radicals are rapidly oxidized, which makes these compounds extremely oxygen sensitive.

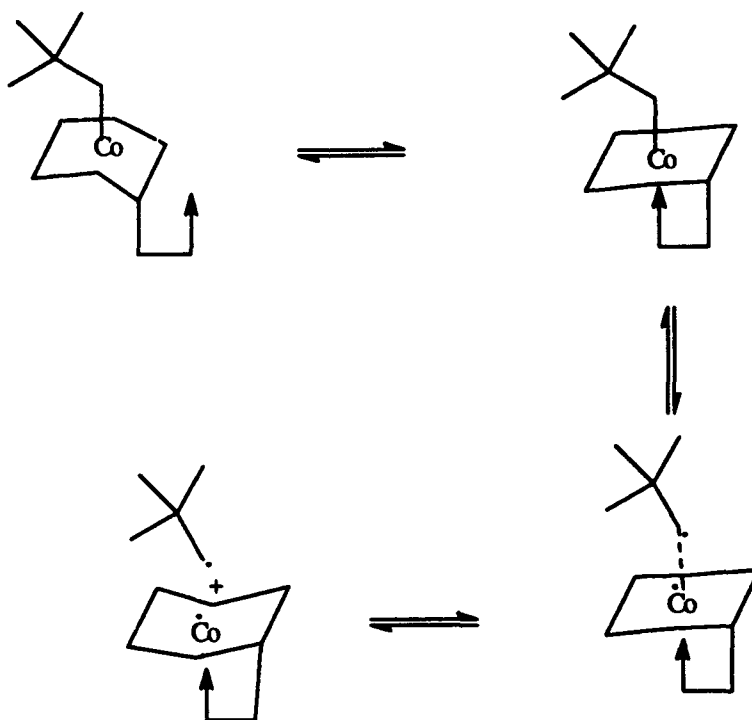


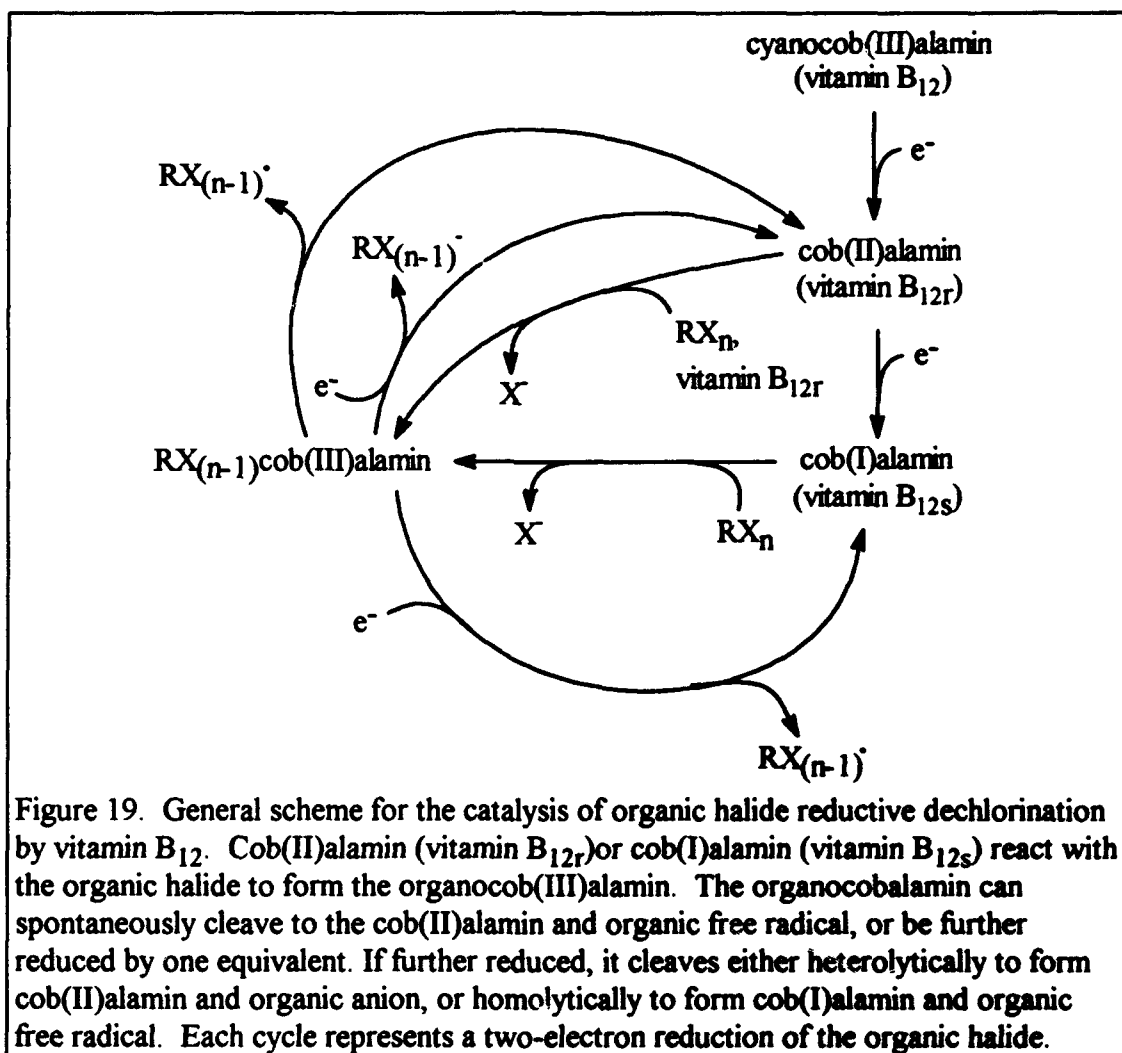
Figure 18. Model of homolytic bond cleavage of sterically strained organocobalamins. Pentavalent cobalt with the cobalamin in the base off configuration can accommodate the bulky organic ligand. When the benzimidazole resumes its attachment to the cobalt α position, homolytic bond cleavage results. (Figure redrawn from Schrauzer and Grate, 1981).

Cobalamins as Catalysts of Reductive Dehalogenation

The potential for cobalamins to catalyze the reductive dehalogenation of alkyl halides was recognized as early as 1964, when alkanes were produced from alkyl halide and vitamin B₁₂ solutions in a controlled potential cell (Hill et al., 1964). Reductive alkylation of cobalamins, followed by reductive dealkylation of the alkylcobalamin, would result in the reductively dechlorinated product. With the advent of bioremediation strategies to mitigate environmental contamination, and efforts to understand and manipulate the biological transformation of environmental pollutants, this aspect of corrinoid chemistry has enjoyed renewed interest. The pesticides mirex and kepone are reductively dechlorinated by vitamin B₁₂ in the presence of a variety of reducing agents (Schrauzer and Katz, 1978). Lindane (1,2,3,4,5,6-hexachlorocyclohexane) is sequentially reductively dechlorinated by vitamin B₁₂ to tetrachlorocyclohexene and eventually to chlorobenzene (Marks et al., 1989; Bieniek et al., 1970). Vitamin B₁₂ will catalyze the sequential reductive dechlorination of carbon tetrachloride to methane (Krone et al., 1989a) and tetrachloroethylene to ethylene (Gantzer and Wackett, 1991). Even the aromatic compounds pentachlorobenzene and pentachlorophenol (Gantzer and Wackett, 1991), and hexachlorobenzene and 2,3,4,5,6-pentachlorobiphenyl have recently been shown to be susceptible to reductive dechlorination by vitamin B₁₂.

I propose a generalized catalytic cycle for the reductive dehalogenation of organic halides by vitamin B₁₂, based upon the above observations (Figure 19). Vitamin B₁₂ (cyanocob(III)alamin) can be reduced to the Co(II) oxidation state to form vitamin B_{12r}, or to the Co(I) oxidation state to form vitamin B_{12s}. Vitamin B_{12s} reacts with alkyl halides by an S_N2 mechanism (Schrauzer and Deutsch, 1969), releasing a free halide anion and forming a σ -bond between the cobalt center and the

carbon which formerly hosted the released halide. Alternatively, two equivalents of vitamin B_{12r} react with an alkyl halide by a free radical mechanism to form the alkylcobalamin (Halpern and Maher, 1965; Blaser and Halpern, 1980). The fate of the organocobalamin is dependent upon the nature of the organic ligand and environmental conditions.



The organocobalamin can cleave spontaneously to the organic free radical and cob(II)alamin, a process catalyzed by exposure to light or heat (Schrauzer et al., 1968). The organocobalamin may be further reduced by one equivalent and then cleave heterolytically to form the organic anion and vitamin B_{12r} (Costa et al., 1971, 1974;

Schrauzer et al., 1972), or homolytically to the organic free radical and vitamin B₁₂s (Hill et al., 1971; Costa et al., 1974).

Chlorinated C₁-hydrocarbons are reductively dehalogenated by corrinoids with either dithiothreitol or Ti(III) citrate as electron donors (Krone et al., 1989b). The rate of dechlorination is inversely proportional to the degree of chlorination. All of the chloromethane congeners can be detected as products or intermediates. Methane is generated from methylcobalamin by reduction with Ti(III) citrate. Surprisingly, the rate of methane formation from CCl₄ with methylcobalamin as catalyst and Ti(III) citrate as electron donor is much greater than the rate of methane generation from methylcobalamin alone (Krone et al., 1989b).

On the basis of these observations, Krone et al., (1989b) proposed three possible reaction mechanisms for the reductive dechlorination of CCl₄ by vitamin B₁₂ with Ti(III) citrate as electron donor (Figure 20). In scheme I, Cbl is reduced by two equivalents of Ti(III) to Cbl(I). This nucleophile displaces a chlorine in CCl₄ by nucleophilic substitution. An analogous mechanism occurs for all of the chloromethane congeners. The chloroalkylcobalamins are further reduced by an additional equivalent of Ti(III), and the resulting radical anion decomposes by either heterolytic or homolytic cleavage to form an organic anion or free radical. The carbanion can react with a free proton to form the reductively dechlorinated product. The free radical can be reduced to the anion by an additional equivalent of Ti(III), or can combine with another free radical to form a side product.

In scheme II, successive chlorines are removed by two electron reductions of the organocobalamin and protonation of the reduced compound, without intermediate release of free intermediates. In both scheme I and scheme II, the penultimate intermediate is methylcobalamin. These schemes cannot, therefore, account for the

Scheme I

1. $[\text{Co}^{\text{III}}] + 2\text{Ti}^{\text{III}} \rightarrow [\text{Co}^{\text{I}}] + 2\text{Ti}^{\text{IV}}$
2. $[\text{Co}^{\text{I}}] + \text{CCl}_4 \rightarrow [\text{Co}^{\text{III}}]\text{CCl}_3 + \text{Cl}^\cdot$
3. $[\text{Co}^{\text{III}}]\text{CCl}_3 + \text{Ti}^{\text{III}} \rightarrow [[\text{Co}^{\text{III}}]\text{CCl}_3]^\cdot + \text{Ti}^{\text{IV}}$
4. $[[\text{Co}^{\text{III}}]\text{CCl}_3]^\cdot \rightarrow \cdot\text{CCl}_3 + [\text{Co}^{\text{II}}] \quad (\text{heterolytic})$
 $\rightarrow \cdot\text{CCl}_3 + [\text{Co}^{\text{I}}] \quad (\text{homolytic})$
5. $[\text{Co}^{\text{II}}] + \cdot\text{CCl}_3 \rightarrow [\text{Co}^{\text{III}}]\text{CCl}_3$
6. $2\cdot\text{CCl}_3 \rightarrow \text{Cl}_3\text{C}\cdot\text{CCl}_3$
7. $\cdot\text{CCl}_3 + \text{Ti}^{\text{III}} \rightarrow \cdot\text{CCl}_3 + \text{Ti}^{\text{IV}}$
8. $\cdot\text{CCl}_3 + \text{H}^+ \rightarrow \text{CHCl}_3$
9. $[\text{Co}^{\text{I}}] + \text{CHCl}_3 \rightarrow [\text{Co}^{\text{III}}]\text{CHCl}_2 + \text{Cl}^\cdot$
10. $[\text{Co}^{\text{I}}] + \text{CH}_2\text{Cl}_2 \rightarrow [\text{Co}^{\text{III}}]\text{CH}_2\text{Cl} + \text{Cl}^\cdot$
11. $[\text{Co}^{\text{I}}] + \text{CH}_3\text{Cl} \rightarrow [\text{Co}^{\text{III}}]\text{CH}_3 + \text{Cl}^\cdot$
12. $[\text{Co}^{\text{III}}]\text{CH}_3 + \text{Ti}^{\text{III}} \rightarrow [[\text{Co}^{\text{III}}]\text{CH}_3]^\cdot + \text{Ti}^{\text{IV}}$
13. $[[\text{Co}^{\text{III}}]\text{CH}_3]^\cdot \rightarrow \cdot\text{CH}_3 + [\text{Co}^{\text{II}}]$
 $\rightarrow \cdot\text{CH}_3 + [\text{Co}^{\text{I}}]$
14. $2\cdot\text{CH}_3 \rightarrow \text{H}_3\text{C}\cdot\text{CCH}_3$
15. $\cdot\text{CH}_3 + \text{Ti}^{\text{III}} \rightarrow \cdot\text{CH}_3 + \text{Ti}^{\text{IV}}$
16. $\cdot\text{CH}_3 + \text{H}^+ \rightarrow \text{CH}_4$

Figure 20. Possible mechanisms for vitamin B₁₂ reductive dehalogenation of CCl₄. All of the reactions account for the formation of methane and chloromethane congeners from CCl₄, but only scheme III explains the more rapid formation of methane from CCl₄ than from methylcobalamin (Krone et al., 1989a).

Scheme II

1. $[\text{Co}^{\text{III}}] + 2\text{Ti}^{\text{III}} \rightarrow [\text{Co}^{\text{I}}] + 2\text{Ti}^{\text{IV}}$
2. $[\text{Co}^{\text{I}}] + \text{CCl}_4 \rightarrow [\text{Co}^{\text{III}}]\text{CCl}_3 + \text{Cl}^\cdot$
3. $[\text{Co}^{\text{III}}]\text{CCl}_3 + 2\text{Ti}^{\text{III}} \rightarrow [\text{Co}^{\text{I}}]\text{CCl}_3 + 2\text{Ti}^{\text{IV}}$
4. $[\text{Co}^{\text{I}}]\text{CCl}_3 \rightarrow [\text{Co}^{\text{II}}]\text{CCl}_2 + \text{Cl}^\cdot$
5. $[\text{Co}^{\text{II}}]\text{CCl}_2 + \text{H}^+ \rightarrow [\text{Co}^{\text{III}}]\text{CHCl}_2$
6. $[\text{Co}^{\text{III}}]\text{CH}_3 + 2\text{Ti}^{\text{III}} + \text{H}^+ \rightarrow [\text{Co}^{\text{III}}] + \text{CH}_4 + 2\text{Ti}^{\text{IV}}$

Scheme III

1. $[\text{Co}^{\text{III}}] + 2\text{Ti}^{\text{III}} \rightarrow [\text{Co}^{\text{I}}] + 2\text{Ti}^{\text{IV}}$
2. $[\text{Co}^{\text{I}}] + \text{CCl}_4 \rightarrow [\text{Co}^{\text{III}}]\text{CCl}_3 + \text{Cl}^\cdot$
3. $[\text{Co}^{\text{III}}]\text{CCl}_3 + 2\text{Ti}^{\text{III}} \rightarrow [\text{Co}^{\text{I}}]\text{CCl}_3 + 2\text{Ti}^{\text{IV}}$
4. $[\text{Co}^{\text{I}}]\text{CCl}_3 + \text{CCl}_4 \rightarrow \text{Cl}_3\text{C}[\text{Co}^{\text{III}}]\text{CCl}_3$
5. $\text{Cl}_3\text{C}[\text{Co}^{\text{III}}]\text{CCl}_3 + \text{H}^+ \rightarrow [\text{Co}^{\text{III}}]\text{CCl}_3 + \text{CHCl}_3$
or $\rightarrow \text{Cl}_3\text{C}[\text{Co}^{\text{III}}] + \text{CHCl}_3$
6. $\text{H}_3\text{C}[\text{Co}^{\text{III}}]\text{CH}_3 + \text{H}^+ \rightarrow [\text{Co}^{\text{III}}]\text{CH}_3 + \text{CH}_4$
or $\rightarrow \text{H}_3\text{C}[\text{Co}^{\text{III}}] + \text{CH}_4$

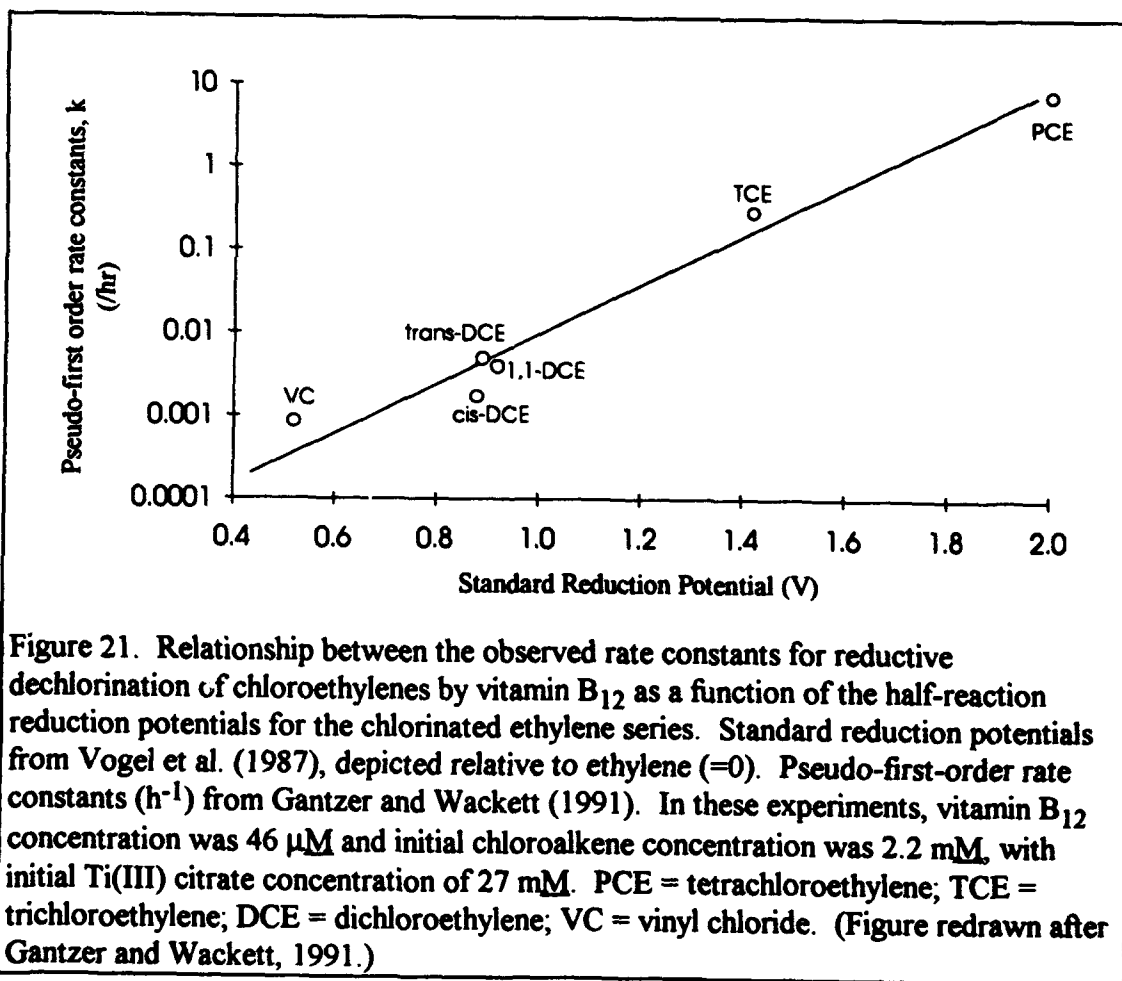
Figure 20, continued.

higher rate of methane formation from CCl_4 by methylcobalamin catalysis than from methylcobalamin itself. In addition, scheme II does not account for the observed free intermediates observed.

Scheme III attempts to account for the increased catalytic activity of methylcobalamin as a consequence of the formation of bis(trichloromethyl)cobalamin. This scheme avoids methylcobalamin as the sole intermediate leading to methane, and also accounts for intermediate dechlorination products. While bis(alkyl)cobalamins

have not been isolated, trans-dialkyl-Co(III) complexes of model compounds have been prepared (Costa et al., 1969, 1971; Farmery and Bush, 1970). This scheme does not, however, account for the recovery of only about 50% of the starting material as gaseous products. This observation would be more satisfactorily explained by free radicals condensing to form products of low volatility.

Vitamin B₁₂ is able to sequentially dechlorinate tetrachloroethylene to ethylene (Gantzer and Wackett, 1991). The rate of dechlorination is directly proportional to the standard reduction potential of the chloroethylene (Vogel et al., 1987). The displacement of a chlorine from the ethylene skeleton reduces the rate of subsequent dechlorination by approximately an order of magnitude (Figure 21).



The dechlorination of hexachloroethane proceeds primarily by a reductive elimination route, producing tetra- and trichloroethylene as the major products (Schanke and Wackett, 1992). As long as both ethane carbons bear a chlorine, reductive elimination is favored. When there are chlorines on one carbon only, reductive dechlorination without elimination occurs. These patterns are observed in the presence of both dithiothreitol and Ti(III) citrate as reducing agents. Lindane (1,2,3,4,5,6-hexachlorocyclohexane) is reductively dechlorinated to tetrachlorocyclohexene and eventually to chlorobenzene (Bieniek et al., 1970; Marks et al., 1989).

Highly chlorinated aromatic compounds are reductively dechlorinated by vitamin B₁₂ (Gantzer and Wackett, 1991; Assaf-Anid et al., 1992). Reductive dechlorinations of perchlorinated aromatic compounds are typically much slower than the corresponding reactions of perchlorinated alkanes; the reactions must be followed for days or weeks rather than minutes or hours. The preferred site of dechlorination is a position *ortho* to two other chlorine atoms (Table 2).

Table 2. Products of reductive dechlorination of aromatic chlorides. The site of dechlorination is preferentially between chlorinated carbons.	
Substrate	Product(s)
Hexachlorobenzene	pentachlorobenzene (Gantzer and Wackett, 1991; Assaf-Anid et al., 1992)
Pentachlorophenol	1,2,3,6- and 1,2,4,6-tetrachlorophenol (Gantzer and Wackett, 1991)
Pentachlorobenzene	1,2,3,5- and 1,2,4,5-tetrachlorobenzene (Assaf-Anid et al., 1992)
2,3,4,5,6-pentachlorobiphenyl	2,3,5,6- and 2,3,4,6-tetrachlorobiphenyl (Assaf-Anid et al., 1992)

Vitamin B₁₂ continues to provide surprises even after 45 years of intensive study. The unique chemistry of this fascinating molecule promises to intrigue chemists for years to come, as well. Originally isolated as "anti-pernicious anemia factor" from

liver, it may hold a key in part to the cure of the pernicious insult of environmental pollution.

Chapter 4

Microbial Reductive Dehalogenation of Organic Halides

The chlorine-carbon bond is not unknown in nature. Marine algae and phytoplankton and some halotolerant terrestrial plants produce an estimated 5×10^6 tons of methyl chloride per year, worldwide (Wuosmaa and Hager, 1990). 2,6-dichlorophenol has even been identified as a sex pheromone of the lone star tick (Berger, 1972). The chlorine-carbon bond is, however, relatively unusual, so few biological mechanisms have evolved to degrade this bond. Chlorinated compounds are thus much longer lived in the environment than are their nonhalogenated counterparts.

Reduction and oxidation processes are crucial to life, for all of life is dependent upon food molecules produced by the reduction of carbon dioxide by photosynthetic or chemolithotrophic organisms. Subsequent oxidation of these compounds releases the

captured chemical energy for use in the life processes of other organisms. Redox processes are also critical to the natural or engineered processing of environmental contaminants.

The ultimate goal of any remediation strategy, whether it involves chemical, biological, or physical processes or any combination of these, should be the complete removal of the contaminant from the

Table 3. Oxidation states of carbon in chlorinated solvents and reference compounds (referenced carbon in boldface).	
Compound	Oxidation State
Methane, CH ₄	-4
Chloroethane, CH ₃ -CH ₂ X	-3
Methyl chloride, CH ₃ X	-2
Chloroethylene, CH ₂ =CHX	-2
Chloroethane, CH ₃ -CH ₂ X	-1
Methylene chloride, CH ₂ X ₂	0
Chloroethylene, CH ₂ =CHX	0
Chloroform, CHX ₃	+2
Perchloroethylene, CX ₂ =CX ₂	+2
Hexachloroethane, CX ₃ -CX ₃	+3
Carbon tetrachloride, CX ₄	+4
Carbon dioxide, CO ₂	+4

contaminated site, without subsequently polluting any other medium. Simple transfer of pollutant from groundwater to air is not acceptable. Complete destruction of the pollutant is therefore desirable, and in the case of organic pollutants, possible. For chlorinated organics, the elements must be chemically or biologically transformed to CO₂, water, and halogen anion.

Chlorinated hydrocarbons can be either oxidized or reduced, depending upon the chemical structure and environmental conditions (Vogel et al., 1987). In general, the relative ease of oxidation is inversely proportional to the degree of chlorination. Chlorination of a hydrocarbon oxidizes the carbon that is chlorinated (see Table 3). For instance, carbon tetrachloride (CT) is already fully oxidized, so that reductive processes are highly favored. Indeed, aerobic (oxidative) biotransformation of CT is unknown.

Surprisingly, however, CO₂ and cellular material are the major products of CT biotransformation under anaerobic conditions, chloroform and other dechlorination products being produced in lesser quantities (Bouwer and McCarty, 1983a, b; Mikesell and Boyd, 1990; Freedman and Gossett, 1991; Egli et al., 1987, 1988; Bouwer and Wright, 1988; Criddle et al., 1990a, b). Transformation to CT is dependent upon the reducing potential of the medium and the bacterium or microbial consortium involved. Sulfate reducing (Cobb and Bouwer, 1991) or denitrifying conditions (Bouwer and McCarty, 1983a, b; Mikesell and Boyd, 1990; Freedman and Gossett, 1991; Egli et al., 1987, 1988; Bouwer and Wright, 1988; Criddle et al., 1990a, b; Cobb and Bouwer, 1991) signal the onset of CT transformation. This activity is apparently due to eubacteria (Freedman and Gossett, 1991) and methanogens (Freedman and Gossett, 1991; Mikesell and Boyd, 1990) which possess the acetyl coenzyme A (acetyl CoA) pathway for C₁ metabolism (Egli et al., 1988). CT transformation activity is also species specific. *Desulfobacterium autotrophicum* transforms CT only to chloroform

and dichloromethane, whereas *Acetobacterium woodii*, grown under the same conditions (fructose fermentation), quickly produces CO₂, followed by assimilation of the CO₂ into acetate, pyruvate, and cellular material (Egli et al., 1988). The acetyl CoA pathway relies upon cobamide-dependent enzymes. *Desulfobacter hydrogenophilus*, which contains no cobamides (Dangel et al., 1987) and utilizes the citric acid cycle, and nitrate reducers, which use the Calvin cycle, do not utilize chlorinated methanes (Egli et al., 1988).

Bacteria can metabolize aromatic hydrocarbons anaerobically by several different mechanisms (Evans, 1977). Only those pathways pertinent to acetogenic and methanogenic soil and sludge bacteria will be discussed here. Adapted anaerobic bacteria first reduce the aromatic ring with hydrogen, followed by non-oxidative ring cleavage to form aliphatic acids which feed into other well-known metabolic pathways. Chlorination of the ring blocks the ring reduction and prevents the transformation of the aromatic substrate (Ferry and Wolfe, 1976).

Non-aryl chlorine atoms can be removed rather non-specifically in a process that can be catalyzed by dead cells and iron porphyrins (Tiedje et al., 1987). Removal of aryl chlorines, on the other hand, demonstrates specificity and adaptation, and requires living cells (Tiedje et al., 1987). Early on, workers noted that the aromatic chlorine was replaced with hydrogen (rather than with, say, hydroxyl), that is, by reductive dechlorination. Reductive dechlorination has been observed in soils, sediments, aquifers, and sewage sludge.

The degradation pattern is dependent upon the starting compound, the microbial consortium, and environmental factors. Dechlorination of chlorobenzoates occurs at the *meta* position preferentially. Iodine and bromine direct less specificity, i.e., *ortho* and *para* positions can be dehalogenated (Horowitz et al., 1983; Tiedje et al., 1987).

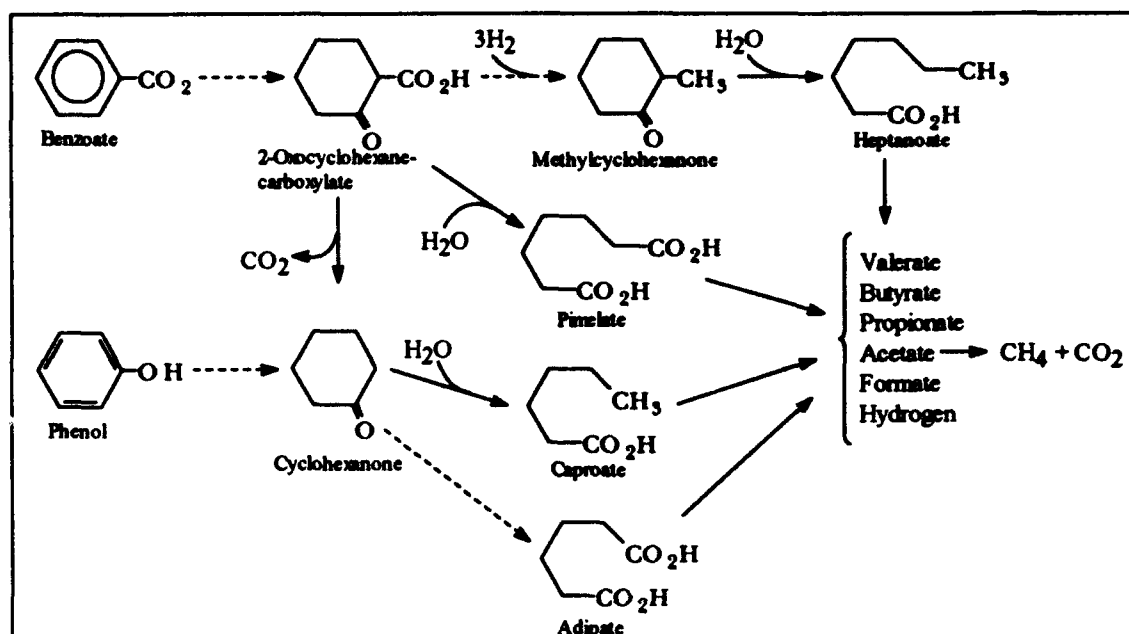


Figure 22. Pathways in the fermentation of benzoate and phenol. These pathways are demonstrated by adapted bacterial consortia from a variety of methanogenic ecosystems (Evans, 1977). Key elements are complete reduction of the ring prior to ring opening, creating aliphatic acids which feed various metabolic pathways.

Rapid dechlorination of chlorobenzoates typically occurs after a 3 week to 6 month lag period. In experiments with mixed feed, dichlorobenzoate inhibited dehalogenation of monochlorobenzoate, i.e., monochlorobenzoate accumulated until dichlorobenzoate was depleted. This inhibition of an enzyme by the first substrate of a pathway is rare.

In contrast, the *ortho* position of chlorinated phenols is preferentially attacked by unacclimated anaerobic bacteria. Relative rates of disappearance from fresh sludge is 2-chlorophenol \gg 3-chlorophenol $>$ 4-chlorophenol (Boyd et al., 1983). An unacclimated upflow anaerobic sludge blanket reactor rapidly removed both *ortho* chlorines from 2,4,6-trichlorophenol. Reductive dechlorination at the 4 position was not observed (Woods et al., 1989).

The reluctance toward *meta* and *para* attack can be overcome by acclimation to the separate monochlorophenols (MCP) or to pentachlorophenol. Two different microbial activities can be induced (Boyd and Shelton, 1984; Mikesell and Boyd, 1986). Acclimation to 2-MCP induces activity toward *ortho* and *para* dechlorination,

while acclimation to 3-MCP induces attack at the *meta* and *para* positions. Acclimation to 4-MCP results in activity toward all positions, and the resulting consortium apparently is a mixture of the bacterial populations induced by 2- or 3-MCP. A mixture of consortia acclimated to each of the individual monochlorophenols will mineralize PCP to CO₂ and CH₄. A summary of known pathways is shown in Figure 23. This figure contains observations from unacclimated and acclimated anaerobic sludges, and from anaerobic soils, sediments, and aquifers. Anaerobic digester sludge acclimated to PCP removes chlorines from all positions of PCP, but the *ortho* chlorines of the less chlorinated congeners are still preferentially removed (Nicholson et al., 1992).

The regiospecificity of chlorophenol reductive dechlorination by unacclimated anaerobic consortia has been correlated with the position of largest negative value for the carbon-chlorine bond charge, as calculated by a Modified Neglect of Differential Overlap (MNDO) method (Cozza and Woods, 1992). This correlation correctly predicts *ortho* dechlorination of chlorophenols and chlorodihydroxyphenols, and *meta* dechlorination of chlorobenzoates. It also correctly predicts two of three known chloroaniline pathways, the only exception being a prediction of *ortho* dechlorination of 2,3,4,5-tetrachloroaniline, while *para* dechlorination is observed. The calculated carbon-chlorine bond charges for the chlorophenol congeners are shown in Table 4.

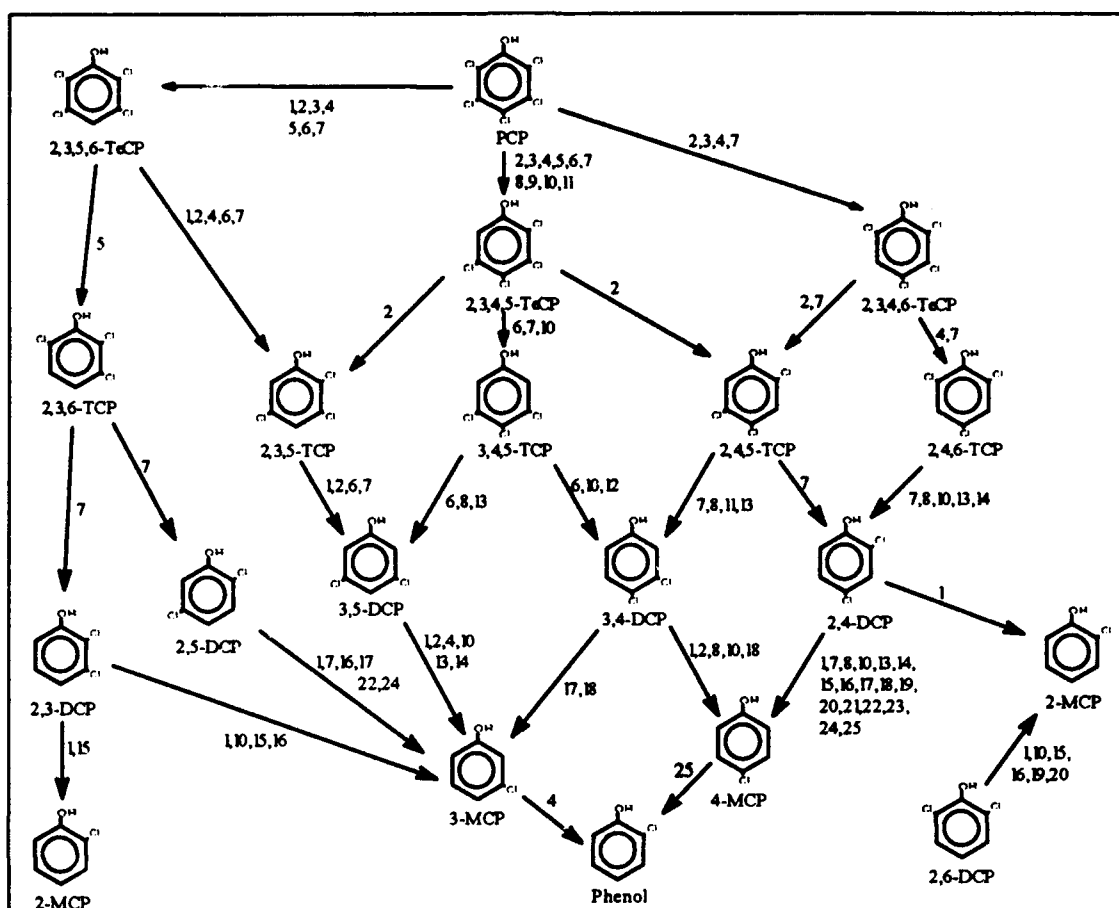


Figure 23. Summary of previously observed chlorophenol reductive dechlorination pathways by acclimated and unacclimated anaerobic consortia. The numbers beside the arrows refer to the following references: 1. Bryant et al., 1991; 2. Ide et al., 1972; 3. Kuwatsuka and Igarishi, 1975; 4. Mikesell and Boyd, 1986; 5. Murthy et al., 1979; 6. Hendriksen et al., 1992; 7. Nicholson et al., 1992; 8. Mikesell and Boyd, 1985; 9. Weiss et al., 1982; 10. Woods et al., 1989; 11. Madsen et al., 1992; 12. Mikesell and Boyd, 1988; 13. Madsen and Aamand, 1992; 14. Mohn and Kennedy, 1992; 15. Hale et al., 1990; 16. Boyd and Shelton, 1984; 17. Gibson and Suffita, 1986; 18. Struijs and Rogers, 1989; 19. Dietrich and Winter, 1990; 20. Haggblom and Young, 1990; 21. Kohring et al., 1989b; 22. Suffita and Miller, 1985; 23. Kohring et al., 1989a; 24. Suffita et al., 1988; 25. Zhang and Wiegand, 1990.

Chapter 5

Comparison of Reactors for Oxygen-Sensitive Reactants

Abstract

Oxygen-sensitive reactants, such as the supernucleophile vitamin B_{12s}, are difficult to study over periods exceeding a few hours due to the pervasion of atmospheric oxygen into the reaction vessel. This paper evaluates and compares three methods for performing such studies. In the first method, purged reactants are sealed in serum vials. In the second method, reactants are hermetically sealed in glass ampoules, in which reducing conditions can be maintained for many months. The third method is a simple reactor system by which vitamin B_{12s} can be maintained for several days while providing ready access for sampling the reaction mixture. Reductive dechlorination of pentachlorophenol by Ti(III) citrate-reduced vitamin B₁₂ was followed using all three methods. Reducing conditions were maintained for up to two months with sealed ampoules. The two-chambered reactor maintained reducing conditions longer than the serum vials when frequent samples were taken.

Introduction

The fully reduced supernucleophile vitamin B_{12s} (cob(I)alamin) has long been known to be oxidized by alkyl halides, resulting in alkylcobalamin. Release and further reduction of the alkyl or alkenyl group yields vitamin B_{12a} and reductively dechlorinated alkane (Schrauzer, 1968). The presence of high quantities of cobalamins in anaerobic bacteria (Dangel et al., 1987; Wolfe, 1985; Zeikus et al., 1985; Krautler et al., 1987, 1988; Stupperich et al., 1988), and the ability of vitamin B₁₂ to catalyze

reductive dehalogenations, has led to speculation that vitamin B₁₂ may serve as an abiotic model of biological reductive dechlorination (Krone et al., 1989b).

Krone et al. (1989a, b), Gantzer and Wackett (1991) and Assaf-Anid et al. (1992) conducted reductive dechlorination experiments using vitamin B₁₂ solutions in a purged serum vial fitted with a septum and crimp sealed. This method is essentially the same as the method developed by Hungate (Hungate, 1950; Macy et al., 1972) and modified by Miller and Wolin (1974) to culture anaerobic bacteria. Reductive dechlorination of chlorinated alkanes proceeds to completion relatively rapidly, so that experiments can be conducted in a few minutes or hours. The reductive dechlorination of aromatic halides, however, is considerably slower than that of polychlorinated alkanes or alkenes (Gantzer and Wackett, 1991; Assaf-Anid et al., 1992), so that experiments with the aromatic compounds require higher reactant concentrations, longer reaction times, or both.

When using septa to exclude oxygen, the choice of septum material is crucial. Miller and Wolin (1974) stipulate that only a butyl rubber septum will exclude oxygen. Krone and co-workers used a Viton septum in their studies of the reductive dechlorination of C₁ compounds by coenzyme F₄₃₀ (Krone et al., 1989a) and corrinoids (Krone et al., 1989b), probably because this material is known to resist penetration by chlorinated methanes. Reactions were followed for at most 2½ hours. In studies by Gantzer and Wackett (1991) and Assaf-Anid et al. (1992), the Viton septum was replaced with Teflon-faced rubber. Both of these studies reported the difficulty of maintaining reduced conditions. Perfusion of atmospheric oxygen through septum punctures or past the seal itself precludes keeping the extreme reducing conditions intact for more than a few hours. Egli et al., (1987) successfully used a second, natural rubber, septum over a Viton septum in their study of reductive

dechlorination of chlorinated methanes by methanogenic bacteria. Since the natural rubber seals more rapidly than the Viton, unwanted gas transfer was minimized.

Assaf-Anid et al. (1992) were able to maintain an amber color in their vitamin B₁₂ solution for several weeks using high concentrations of dithiothreitol as the reductant, but the amber color indicates the less reduced and less oxygen-sensitive form of the cobalamin, vitamin B_{12r} (Pratt, 1972). Maintaining the much more oxygen-sensitive vitamin B_{12s} requires greater diligence. Higher vitamin B₁₂ concentrations and/or frequent sampling can further decrease the time of reducing conditions. Unless one rigorously excludes residual oxygen from commercial compressed gases, constant blanketing of or purging the reaction medium with purified nitrogen or argon does not significantly extend the useful time of reducing conditions (Ljungdahl and Wiegel, 1986).

Our goal was to investigate the reductive dechlorination of chlorophenols catalyzed by vitamin B₁₂ in the presence of Ti(III) citrate. Our initial frustration in maintaining reducing conditions for several weeks in serum vials led us to investigate alternative methods for conducting the experiments. In this paper we describe three simple methods for maintaining reducing conditions for days or months. These methods were used to study the ability of vitamin B_{12s} to reductively dechlorinate pentachlorophenol and 2,3,5-trichlorophenol.

Materials and Methods

All reactions were conducted in 0.66 M Tris (enzyme grade, Life Technologies, Inc., Gaithersburg, MD) buffer, pH 8.2. Vitamin B₁₂ was obtained from Sigma Chemical Company (St. Louis, MO). Pentachlorophenol (PCP, 99.9% purity) was obtained from Sigma Chemical Company, 2,3,5-trichlorophenol from Aldrich Chemical Company (Milwaukee, WI), and 2,3,4,6- and 2,3,5,6-tetrachlorophenol (TeCP) were

purchased from Ultra Scientific, Inc. (N. Kingston, RI). Stock solutions were prepared by dissolving the chlorophenols in methanol. The stock solutions were diluted in the Tris buffer for transfer into the reaction mixtures.

A stock solution of 250 mM Ti(III) citrate in 0.66 M Tris buffer was prepared by a modification of the method of Zehnder and Wuhrman (1976). Thirty mL of 13% TiCl_3 in 20% HCl (Fluka Chemical Corp., Ronkonkoma, NY) was added to a slurry of 8.0 g Tris and 14.7 g Na_3 citrate (Mallincrodt, Inc., St. Louis, MO) in about 40 mL distilled, deionized water. During preparation, the solution was kept in an ice bath and was constantly sparged with argon. The pH was adjusted to 8.2 by the addition of NaOH flakes. The solution was then diluted to 100 mL with distilled, deionized water. This stock solution was divided into 15 mL aliquots and stored frozen in serum vials capped with Teflon-lined rubber septa and aluminum crimp seals. The frozen stock solutions maintained potency for at least five months. Upon thawing, however, the solution slowly loses potency as atmospheric O_2 perfuses through the septum.

Two-chambered reactor

To prepare the two-chambered batch reactor (TCR), a narrow neck was introduced into a glass culture tube in a flame, creating two separate chambers (Figure 24). The neck was just wide enough to allow insertion of a flea-sized stir bar. With the reaction mixture in the bottom of the tube, the upper chamber was sealed with a Teflon-lined rubber septum and screw-top septum cap. The gas train consisted of copper tubing fitted to a source of compressed argon. The gas passed through a flow meter, O_2 scavenging cartridge (OMI-1, Supelco, Inc., Bellefonte, PA) and needle valve. At the end of the copper train, a length of fused silica capillary tubing (0.32 mm I.D. gas chromatograph guard column, J&W Scientific, Folsom, CA) was attached with compression fittings. The capillary was used as a long needle and could be passed through the septum into the liquid to purge the reaction medium, or raised to flush the

upper chamber. Gas pressure was vented with a second capillary that also served as a sampling port. When extended into the reaction mixture, samples were forced out under the pressure of the purging gas.

Additions of reagents directly to the lower chamber were conveniently made with a capillary syringe (J&W Scientific, Folsom, CA). The standard Teflon ferrule and aluminum spacer were drilled to 0.5 mm to accommodate a length of 0.32 mm I.D. fused silica capillary tubing (0.5 mm O.D.).

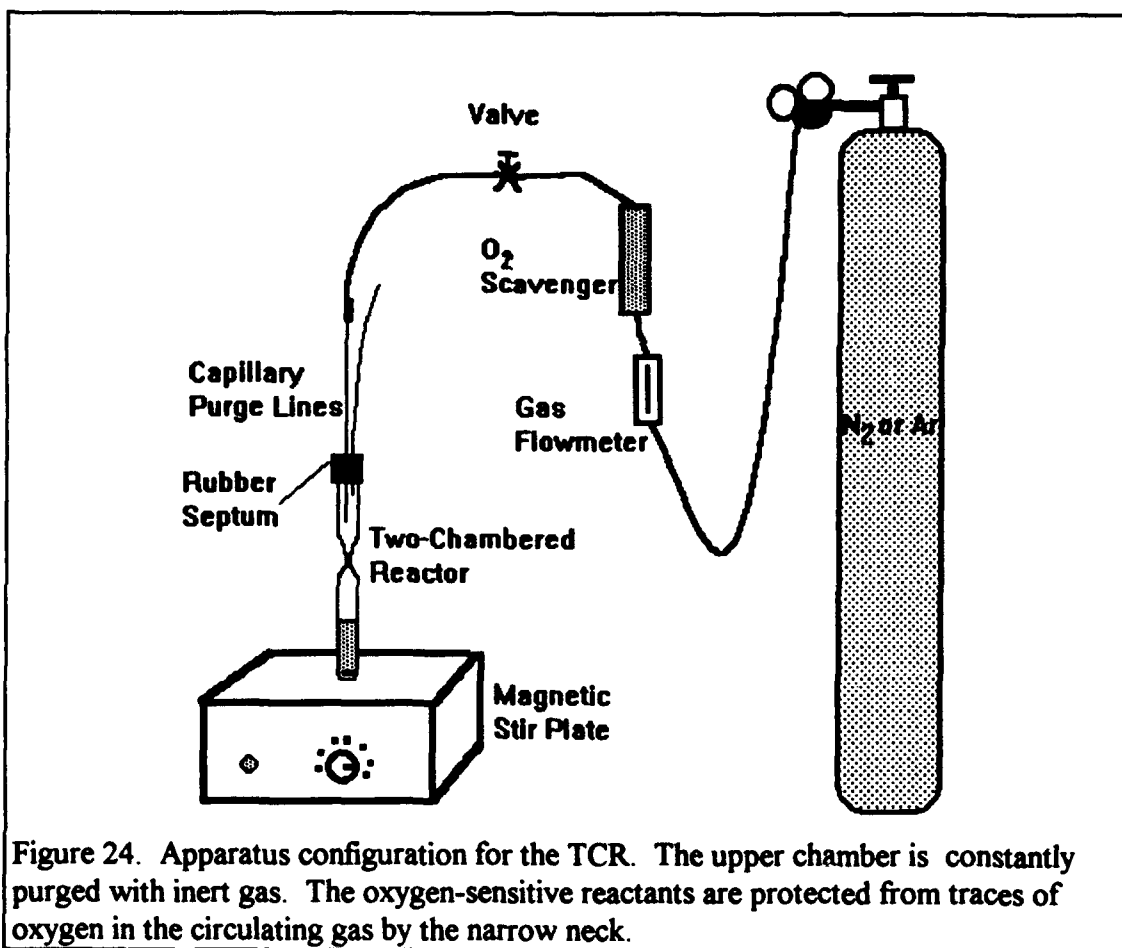


Figure 24. Apparatus configuration for the TCR. The upper chamber is constantly purged with inert gas. The oxygen-sensitive reactants are protected from traces of oxygen in the circulating gas by the narrow neck.

TCR technique

A TCR was covered with aluminum foil to exclude light. Three mL of a vitamin B₁₂ solution were placed in the lower chamber and the solution was purged for 15 minutes with argon at approximately 15 mL/min flow rate. Three hundred μ L of stock Ti(III) citrate were added by means of the capillary syringe. After allowing 15 minutes for complete reduction of the vitamin B₁₂, the end of the purging capillary was raised into the upper chamber of the TCR and 30 μ L of PCP stock solution were added directly to the reaction mixture by capillary syringe to begin the reaction. Final concentrations were 9.0×10^{-4} M vitamin B₁₂, 0.023 M Ti(III) citrate, and 9.0×10^{-6} M PCP. Samples were periodically collected for analysis through the venting capillary.

Ampoule technique

One mL aliquots of a solution containing vitamin B₁₂ and PCP were dispensed to several 2 mL glass ampoules (Wheaton Scientific, Millville, NJ). Each ampoule was in turn sparged for five minutes with argon at approximately 15 mL/min flow rate. One hundred μ L of stock Ti(III) citrate were then added, and the ampoule sealed in a flame. Final concentrations were 9.0×10^{-4} M vitamin B₁₂, 0.023 M Ti(III) citrate, and 9.0×10^{-6} M PCP. Within five minutes the originally red solution turned a deep blue, indicating reduction to vitamin B_{12s}. The ampoules were placed in a dark incubator at 27°. At intervals, an ampoule was sacrificed and the contents assayed for chlorophenols.

A similar set of ampoules was prepared to screen for the ability of vitamin B₁₂ to reductively dechlorinate 2,3,5-trichlorophenol. Initial concentrations of reactants were 1.5×10^{-4} M 2,3,5-TCP, 5.4×10^{-4} M vitamin B₁₂, and 7.3×10^{-3} M Ti(III) citrate.

Serum vial technique

The serum vial method of Krone et al. (1989a, b), was investigated using several different types of septa. As a screening procedure, 3 mL of 5×10^{-4} M vitamin B₁₂

were placed in 10 mL clear serum vials and capped with various septa and aluminum crimp seals. Septa tested were Teflon/rubber, Viton, and butyl rubber (Wheaton). The solutions were purged for at least 15 minutes with argon using the same gas train described above. It was necessary to pierce some of the septa with hypodermic needles to insert the purging capillaries. After the purging was complete, 100 μ L of stock Ti(III) citrate was added by syringe to reduce the vitamin B₁₂. The vials were monitored for maintenance of reducing conditions.

A separate serum vial was prepared with 3 mL of vitamin B₁₂ solution. The vial was capped with a butyl rubber septum and aluminum crimp seal, and covered with aluminum foil to exclude light. Following 15 minutes of argon purge, Ti(III) citrate stock solution was added by syringe. After allowing 15 minutes for complete reduction of the vitamin B₁₂, PCP stock solution was added directly to the reduced vitamin B₁₂ solution to begin the reaction. Final concentrations were 9.0×10^{-4} M vitamin B₁₂, 0.023 M Ti(III) citrate, and 9.0×10^{-6} M PCP. Samples were periodically collected by syringe for analysis. The reaction mixture was mixed throughout the experiment with a magnetic stir bar.

Analytical methods

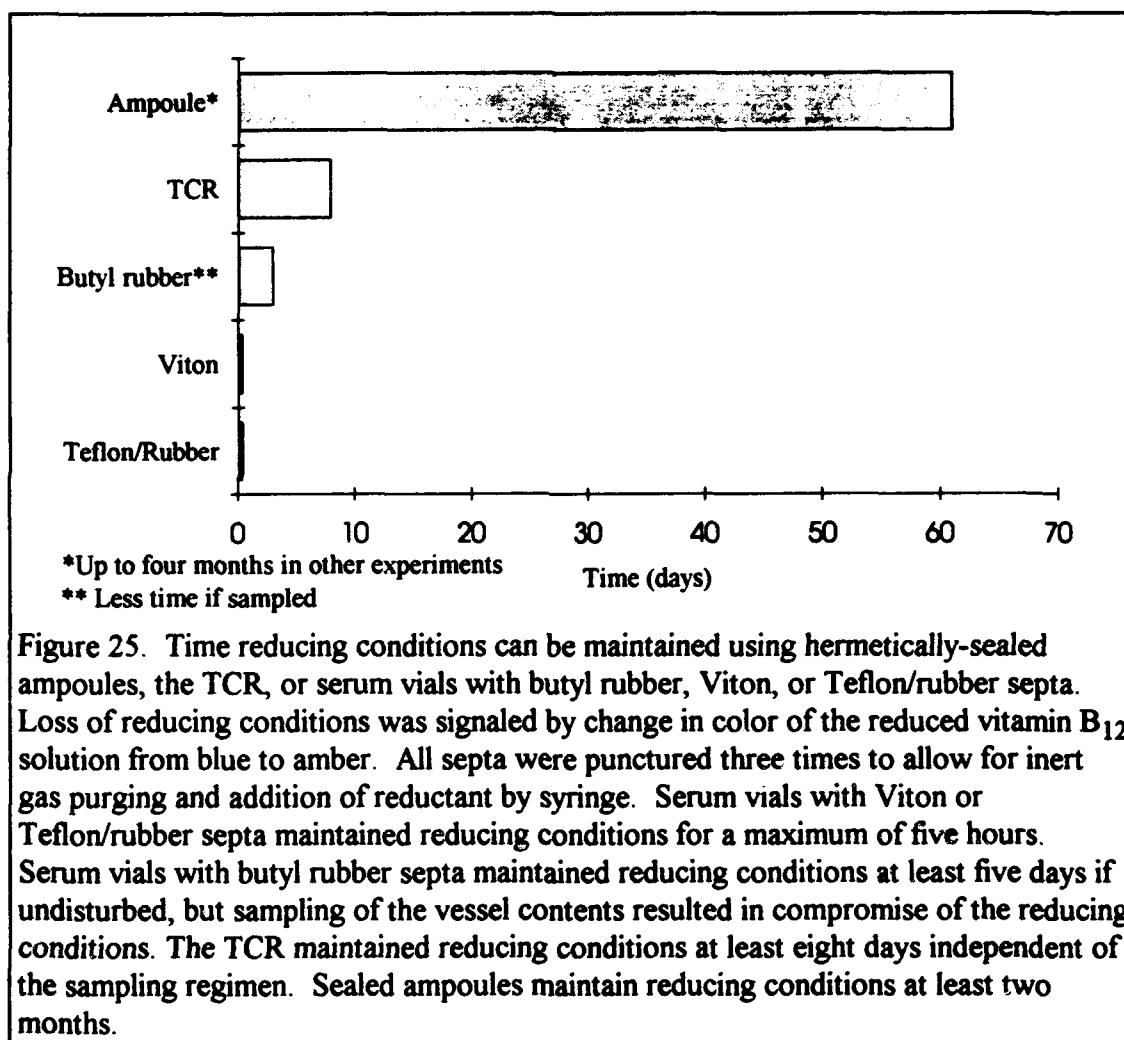
Chlorophenol concentrations were determined by gas chromatography. Chlorophenols were acetylated and extracted with hexane using a modification of the procedure developed by Voss et al. (1980) and Perkins (1992). One-hundred μL samples were mixed in a screw-top culture tube with exactly one mL of a reaction medium containing 43 g/L K_2CO_3 (Mallinckrodt) and one mg/L 2,4,6-tribromophenol (Aldrich) as internal standard. One-hundred μL of acetic anhydride (Aldrich) was added, the tube was capped with a Teflon-lined cap, and shaken on a wrist-action shaker for 20 minutes. Three mL of hexane (EM Science, Gibbstown, NJ) were added, and the tube shaken for an additional 10 minutes. The hexane fraction was transferred to an autosampler vial and capped with a Viton septum and crimp-seal cap. Gas chromatography was performed on the hexane extracts with a Hewlett-Packard model 5890A gas chromatograph equipped with an electron capture detector and fitted with a J&W Scientific DB-5 30 m \times 0.32 mm I.D. column. Injector and detector temperatures were 250 and 320°C, respectively. A one μL aliquot was introduced by splitless injection. Initial oven temperature was 45°, which was held for two minutes, followed by a 15°/min ramp to 105° and a 5°/min ramp to 215°, which was held for 5 minutes. The carrier was helium (1 mL/min), and the makeup gas was argon-methane (95%-5%, 60 mL/min).

Results

Maintenance of reducing conditions

In the serum vial septum screening test, reducing conditions were considered to be lost when the blue solution of vitamin $\text{B}_{12\text{s}}$ turned the amber color of vitamin $\text{B}_{12\text{r}}$. Figure 25 compares the duration of reducing conditions maintained by each septum. Using the serum vial method, only butyl rubber septa maintained reducing conditions

for greater than a few hours, but even this performance was limited by sampling the contents. An undisturbed serum vial with a butyl rubber septum maintained reducing conditions for five days, but the solution turned amber after as few as four 100 μ L syringe samples of vessel contents were removed. This occurred even when the vessel was initially pressurized with the purging gas.



The TCR maintained reducing conditions for up to eight days, independent of the number of samples taken from the vessel. An identical culture tube, purged with argon but without the constriction, maintained reducing conditions for only a few hours.

The hermetically sealed ampoules provided the longest useful time of reducing conditions. The last ampoule in the 2,3,5-TCP experiment was still blue when it was sacrificed after 59 days. Other ampoules in our laboratory have maintained reducing conditions for as long as four months. These ampoules can be destructively sampled by breaking the glass seal.

Reductive dechlorination of chlorophenols

Figure 26 shows the progress of the reductive dechlorination of PCP conducted in the TCR, sealed ampoules, and butyl rubber-sealed serum vials. Products in all cases, not shown, were 2,3,4,6- and 2,3,5,6-tetrachlorophenol. Similar rates were seen in all cases. The rate of reductive dechlorination in the TCR was slightly higher than in the vial or ampoule. Conversion of PCP was greater than 95% after 45 hours.

Reductive dechlorination continued in the ampoules until the PCP was virtually gone at

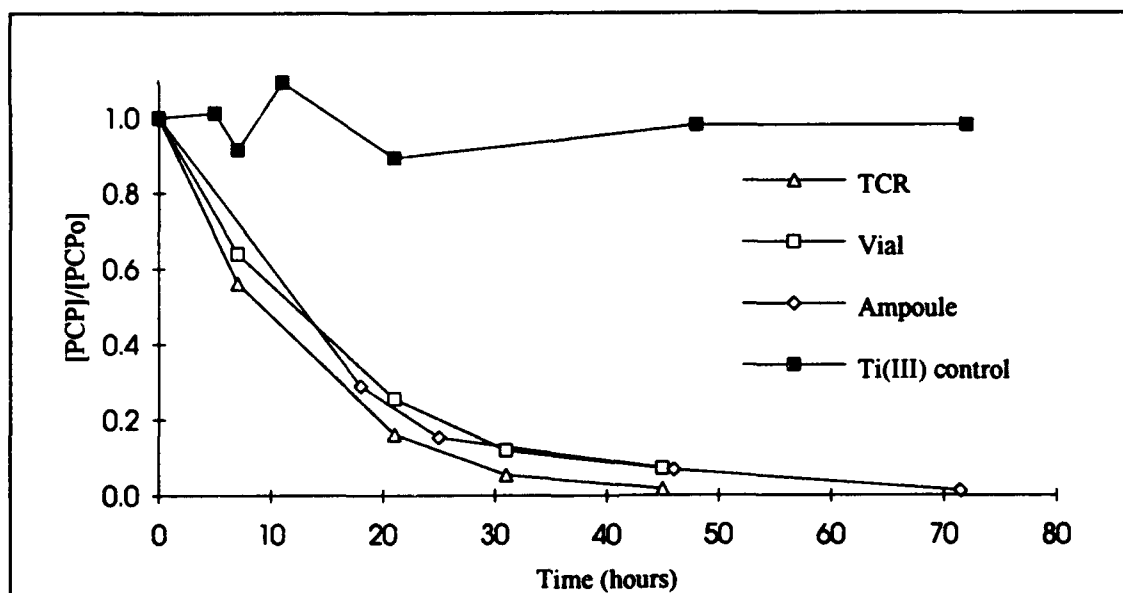


Figure 26. Progress curves for reductive dechlorination of PCP. Initial concentrations were 1×10^{-5} M PCP and 1×10^{-3} M vitamin B₁₂ with excess Ti(III) citrate in either the TCR, butyl rubber-sealed serum vial, or hermetically-sealed ampoule. Ti(III) citrate control values were obtained from a TCR experiment.

72 hours. The rate of reductive dechlorination in the butyl rubber-capped vial was the same as that in the ampoules, but decreased toward the end of the run coincident with a change of solution color to reddish brown. With the great excess of vitamin B₁₂ present, the reactions were seen to be first order in chlorophenol. Pseudo-first order rate constants determined by linear regression of the $-\ln([PCP]/[PCP_0])$ vs. time data were 0.090, 0.062, and 0.067 hr⁻¹ for the TCR, ampoule, and serum vial runs, respectively. The rates in the ampoules and serum vials were not statistically different ($p = 0.15$), while the increased rate observed in the TCR was statistically significant ($p < 0.0001$) (Appendix D)

Figure 27 shows the progress of reductive dechlorination of 2,3,5-TCP conducted in sealed ampoules. Vitamin B₁₂s, evidenced by the persistence of deep blue colored solution, was maintained for 59 days. Although the last ampoule was sacrificed at 59 days, the vitamin B₁₂ solution was still blue, and reducing conditions probably could have been maintained much longer. The dechlorination product was primarily 2,5-dichlorophenol. A control reaction, which contained the same concentration of 2,3,5-TCP and Ti(III) citrate, but no vitamin B₁₂, showed no loss of TCP in 31 days. Some of the prepared ampoules failed to maintain vitamin B₁₂s, probably due to an imperfect seal. Only one of the Ti(III) citrate controls maintained reducing conditions for an extended period, as indicated by the light blue color of Ti(III). This ampoule was sacrificed for analysis on day 31.

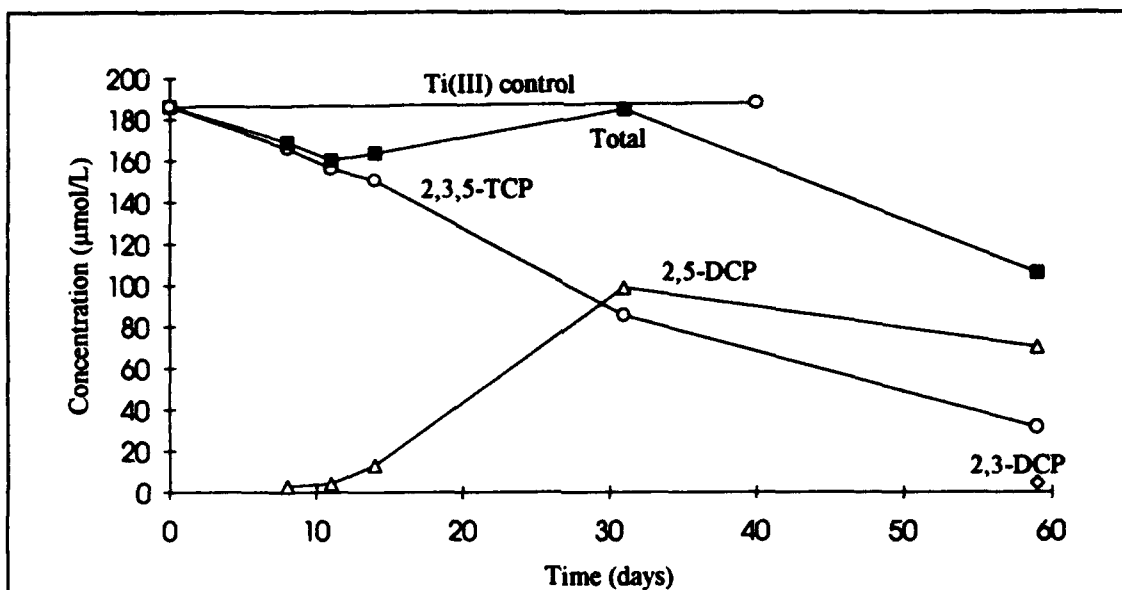


Figure 27. Progress curve for the reductive dechlorination of 2,3,4-TCP by vitamin B₁₂. Initial concentrations were 1.5×10^{-4} M 2,3,5-TCP and 5.4×10^{-4} M vitamin B₁₂, with excess Ti(III) citrate, in ampoules. The deep blue color of vitamin B₁₂ persisted throughout the course of the reaction. The primary product was 2,5-dichlorophenol. Ti(III) citrate at the same concentration, but in the absence of vitamin B₁₂, did not reductively dechlorinate 2,3,5-TCP.

Discussion

Figure 26 shows similar rates of reductive dechlorination using the TCR, the serum vial, and sealed ampoules. In all three reactors, reductive dechlorination proceeded as long as the reaction medium remained blue. Slow perfusion of atmospheric oxygen is effectively scavenged by the excess reducing agent. Toward the end of the serum vial run, the vitamin B₁₂ solution changed to a reddish brown color, and reductive dechlorination ceased.

The difference in rate demonstrated by the TCR method in comparison to the serum vial and ampoule methods is difficult to explain. The TCR was probably better mixed throughout the reaction than were the ampoule or the serum vial. The ampoule was incubated with no mixing, while the serum vial was mixed with a stir bar. The convex bottom of the serum vial and the small size of the stir bar may have contributed

to imperfect mixing. There was a slight vortex in the reaction mixture in the TCR, indicating efficient mixing by the flea-sized stir bar used in this reactor. Mixing, however, should only increase the rate of a diffusion-controlled reaction, and only when the reactants are first contacted. It should have no effect on a reaction in a homogeneous mixture in a batch reaction.

The ampoule method is very simple and lends itself to screening experiments and the study of reactions that may take weeks or even months to produce observable change. Oxygen perfusion is prevented by permanently sealing off any access to atmospheric oxygen. We have been able to maintain reducing conditions, as determined by persistence of the blue color of vitamin B₁₂s, for at least two months. Kinetic studies can be accomplished by destructively sampling carefully-prepared replicate solutions.

The serum vial technique is most useful for volatile substrates that react relatively rapidly. Butyl rubber septa maintain reducing conditions most effectively, but may not be compatible with the substrate under study (Krone, 1989a, b).

The TCR is especially suitable for kinetic studies with nonvolatile substrates, as successive samples of the same reaction mixture can be taken without changing the reaction conditions. Traces of atmospheric oxygen which perfuse around the septum or through syringe puncture wounds are flushed out of the upper chamber before they can diffuse into contact with the oxygen-sensitive reaction mixture. The narrow neck prevents convective transfer of oxygen by the circulating purge gas. Oxygen flux to the surface of the reaction medium is therefore limited to diffusion between the chambers through the neck. This diffusive transfer would be expected to be very slow, due to the small cross section of the neck and small oxygen concentration gradient between the upper and lower chambers. The TCR has the additional advantage of permitting

efficient mixing of the reaction mixture without risking contact of the reactants with septum material.

Conclusions

We have compared three techniques for conducting oxygen-sensitive reactions. By these methods, relatively slow reactions can conveniently be studied. The TCR is a new method not previously encountered in reductive dechlorination studies. As the ability of vitamin B₁₂ to reductively dechlorinate chlorinated hydrocarbons is extended to nonvolatile substrates, its use in kinetic studies will be valuable. Serum vials are most suitable for volatile substrates which react relatively rapidly. Careful consideration of septum material must be made when using serum vials for these studies. Sealed ampoules can maintain extreme reducing conditions for extended periods and should prove useful in screening the ability of vitamin B₁₂ to reductively dechlorinate ever more exotic substrates. We have used these methods to demonstrate the reductive dechlorination by vitamin B₁₂s of PCP and 2,3,5-TCP.

Chapter 6

Regiospecificity of Chlorophenol Reductive Dechlorination by Vitamin B₁₂s

Abstract

Vitamin B₁₂, reduced by titanium(III) citrate to the supernucleophile vitamin B₁₂s, catalyzes the reductive dechlorination of chlorophenols. The observed regiospecificity is consistent with a mechanism whereby the nucleophile first displaces a chlorine by an addition-elimination mechanism, followed by cleavage of the Co-C bond. Addition of a proton results in a reductively dechlorinated arene. Reaction of various chlorophenols with vitamin B₁₂ favored reductive dechlorination at positions *ortho* to another chlorinated carbon, except for chlorines *ortho* to the hydroxyl group. This chlorine is particularly resistant to reductive replacement. This resulted in a reductive dechlorination pattern favoring removal of *para* and *meta* chlorines, in contrast to the regiospecificity exhibited by unacclimated anaerobic microbial consortia.

Introduction

Natural and engineered biological systems can transform environmental pollutants into less noxious agents. A thorough understanding of the biochemical reactions involved in these transformations may lead to improved processes for bioremediation of hazardous waste sites. A transformation of particular interest is reductive dechlorination, as a large number of hazardous substances of environmental concern are chlorinated hydrocarbons.

Pentachlorophenol (PCP) has been used for many years as a pesticide in rice culture, and as a wood preservative. In addition, other chlorinated hydrocarbons of environmental concern, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-

trichlorophenoxyacetic acid (2,4,5-T), are transformed by anaerobic bacteria to chlorophenols (CPs) (Mikesell and Boyd, 1985). As a result of their extensive use, CPs are widely distributed in the environment, and are significant contaminants at many sites selected for cleanup on the National Priorities List of the Superfund program.

Since the discovery of reductive dechlorination products in soil heavily contaminated with PCP (Ide et al., 1972), a number of studies have collectively led to a more thorough description of the biological transformation of CPs. Anaerobic biodegradation of CPs occurs by a process of reductive dechlorination, whereby the carbon to which a chlorine is attached is reduced while the chlorine is exchanged for a hydrogen. Reductive dechlorination has been observed in soils, sediments, aquifers, and sewage sludge. The degradation pattern depends upon the starting compound, the microbial consortium, and environmental factors. Unacclimated anaerobic consortia dechlorinate PCP preferentially at the position *ortho* to the phenolic group, a pattern which has been correlated with the position of largest negative value for the carbon-chlorine bond charge (Cozza and Woods, 1992). Anaerobic digester sludge acclimated to PCP removes chlorines from all positions of PCP, but the *ortho* chlorines of the less chlorinated congeners are still preferentially removed (Nicholson et al., 1992).

The dechlorination pattern can also be altered by specific acclimation of a consortium to different monochlorophenols (MCP) (Boyd and Shelton, 1984; Mikesell and Boyd, 1986). Two different microbial activities can be induced. Acclimation to 2-MCP induces activity toward *ortho* and *para* dechlorination, while acclimation to 3-MCP induces attack at the *meta* and *para* positions. Acclimation to 4-MCP results in activity toward all positions, and the resulting consortium apparently is a mixture of the bacterial populations induced by 2- or 3-MCP. A mixture of consortia acclimated to each of the individual monochlorophenols will mineralize PCP to CO₂ and CH₄. Figure 28 shows the known pathways for CP reductive dechlorination, summarizing

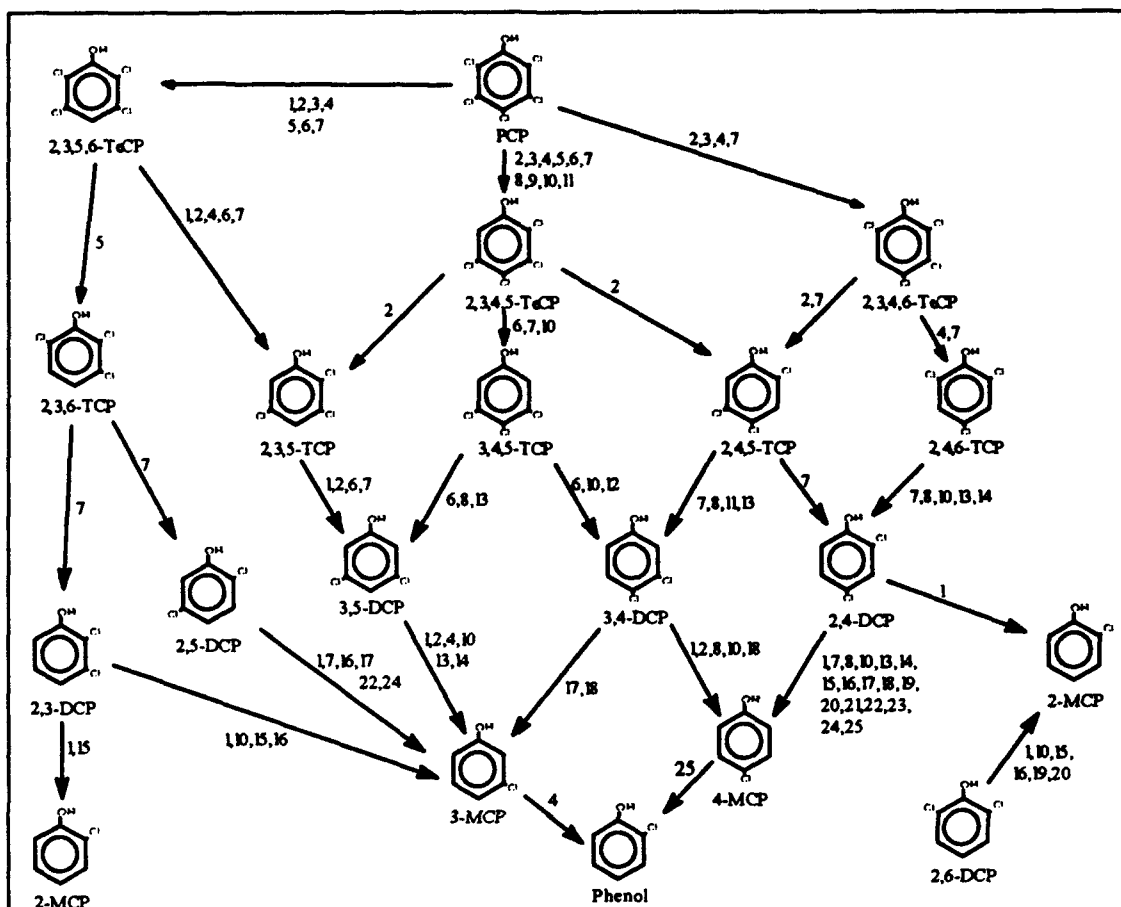


Figure 28. Summary of previously observed chlorophenol reductive dechlorination pathways by acclimated and unacclimated anaerobic consortia. The numbers beside the arrows refer to the following references: 1. Bryant et al., 1991; 2. Ide et al., 1972; 3. Kuwatsuka and Igarishi, 1975; 4. Mikesell and Boyd, 1986; 5. Murthy et al., 1979; 6. Hendriksen et al., 1992; 7. Nicholson et al., 1992; 8. Mikesell and Boyd, 1985; 9. Weiss et al., 1982; 10. Woods et al., 1989; 11. Madsen et al., 1992; 12. Mikesell and Boyd, 1988; 13. Madsen and Aamand, 1992; 14. Mohn and Kennedy, 1992; 15. Hale et al., 1990; 16. Boyd and Shelton, 1984; 17. Gibson and Suflita, 1986; 18. Struijs and Rogers, 1989; 19. Dietrich and Winter, 1990; 20. Haggblom and Young, 1990; 21. Kohring et al., 1989b; 22. Suflita and Miller, 1985; 23. Kohring et al., 1989a; 24. Suflita et al., 1988; 25. Zhang and Wiegel, 1990.

observations from unacclimated and acclimated anaerobic sludges, and from anaerobic soils, sediments, and aquifers.

Several naturally-occurring metalloorganic compounds have been shown to catalyze reductive dechlorination, and the implications to natural processes have been recognized. Carbon tetrachloride, perchloroethylene and their dechlorinated congeners are reductively dechlorinated by the cobalt corrinoid vitamin B₁₂, the nickel porphinoid coenzyme F₄₃₀, and the iron porphyrin heme, with titanium(III) citrate or dithiothreitol as electron donor (Krone et al., 1989a, b; Gantzer and Wackett, 1991). Vitamin B₁₂ can also catalyze the reductive dechlorination of the pesticides lindane (Bienie et al., 1970; Marks et al., 1989), mirex and kepone (Schrauzer and Katz, 1978), and the aromatic halides hexachlorobenzene and pentachlorobenzene (Gantzer and Wackett, 1991; Assaf-Anid et al., 1992) pentachlorophenol (Gantzer and Wackett, 1991), and 2,3,4,5,6-pentachlorobiphenyl (Assaf-Anid et al., 1992).

Anaerobic bacteria are rich in the metalloorganic cofactors which catalyze reductive dehalogenation. Acetogenic and methanogenic bacteria are especially rich in corrinoids, both cytoplasmic and membrane bound (Dangel et al., 1987; Zeikus et al., 1985; Wolf, 1985; Krautler et al., 1987, 1988; Stupperich et al., 1988). Some of these corrinoids have unique functional moieties with as yet undetermined influence on the chemical activity of the molecule (Stupperich et al., 1988).

In contrast to the regiospecificity of reductive dechlorination expressed by anaerobic bacteria, Gantzer and Wackett (1991) reported that, under the conditions of their study, vitamin B₁₂ transformed only about 12% of the PCP, and only the *meta* and *para* positions were dechlorinated. The regiospecificity of reductive dechlorination shown by unacclimated and acclimated anaerobic microbial consortia and by vitamin B₁₂ invites comparison. In this study, we examine the reductive dechlorination of all of

the penta-, tetra-, and trichlorophenols by vitamin B₁₂ and compare the pattern observed to that of anaerobic microbial consortia.

Materials and Methods

All reactions were conducted in 0.66 M Tris (Life Technologies, Inc., Gaithersburg, MD) buffer, pH 8.2. Sources of reagents were as follows: Vitamin B₁₂ and PCP (99.9% purity) were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,4,5-, 2,3,4,6- and 2,3,5,6-tetrachlorophenols (TeCP) were purchased from Ultra Scientific, Inc. (N. Kingston, RI.), 95+% purity. All other CPs were purchased from Aldrich Chemical Co. (Milwaukee, WI), and were 98-99% purity. A stock solution of 250 mM Ti(III) citrate in 0.66 M Tris buffer, pH 8.2, was prepared from Na₃ citrate (Mallincrodt, Inc., St. Louis, MO) and TiCl₃ solution (Fluka Chemical Corp., Ronkonkoma, NY) as previously described (Chapter 5). Stock solutions of the CPs were made in methanol and diluted in Tris buffer for transfer to the reaction mixtures.

Reduction of vitamin B₁₂ by Ti(III) citrate

The ability of Ti(III) citrate to reduce vitamin B₁₂ to the fully-reduced vitamin B_{12s} was examined. A spectrophotometer cell containing a solution of 5×10^{-5} M vitamin B₁₂ was fitted with a thick rubber septum. The solution was purged with N₂, and Ti(III) citrate solution was added to a final concentration of 8×10^{-3} M. The progress of the reduction was followed on a Hewlett-Packard model 8452A phased array spectrophotometer operated in the kinetics mode.

Reductive dechlorination of chlorophenols

Reductive dechlorination of PCP was conducted in a two-chambered reactor (TCR, Chapter 5). Initial concentrations of reactants were 4.3×10^{-6} M PCP, 4.8×10^{-4} M vitamin B₁₂, and 0.008 M Ti(III) citrate. Samples were taken periodically for analysis of chlorophenols. A control reaction with PCP and elevated Ti(III) citrate in

buffer was conducted in a TCR. Initial concentrations were 7.7×10^{-6} M PCP and 0.023 M Ti(III) citrate.

All other reactions were conducted in hermetically-sealed 2 mL glass ampoules (Wheaton Scientific, Millville, NJ) containing one mL of reaction mixture. The reaction mixtures contained 5×10^{-4} M vitamin B₁₂ and 8×10^{-3} M Ti(III) citrate in Tris buffer. The initial concentrations of CPs ranged from 3.5×10^{-6} M for PCP to 1.9×10^{-4} M for 2,3,5-TCP. The ampoules were incubated in the dark at 27°C. Replicate ampoules were periodically sacrificed for assay of the CPs transformed and produced.

Analytical method

The fate of the chlorophenol reactants was followed by gas chromatography using a modification of a miniaturized version (Perkins, 1992) of the acetylation procedure of Voss et al. (1980). One-hundred μ L samples were mixed in a screw-top culture tube with exactly one mL of a reaction medium containing 43 g/L K₂CO₃ and one mg/L 2,4,6-tribromophenol as internal standard. One-hundred μ L of acetic anhydride was added, the tube was capped with a Teflon-lined cap, and shaken on a wrist-action shaker for 20 minutes. Three mL of hexane were then added, and the tube shaken for an additional 10 minutes. The hexane fraction was transferred to an autosampler vial and capped with a Viton septum and crimp-seal cap. Gas chromatography was performed on the hexane extracts with a Hewlett-Packard model 5890A gas chromatograph equipped with an electron capture detector and fitted with a J&W Scientific DB-5 30 m \times 0.32 mm I.D. column. Injector and detector temperatures were 250 and 320°C, respectively. A 1- μ L aliquot was introduced by splitless injection. Initial oven temperature was 45°C, which was held for two minutes, followed by a 15°/min ramp to 105° and a 5°/min ramp to 215°, which was held for 5

minutes. The carrier was helium (1 mL/min), and the makeup gas was argon-methane (95%-5%, 60 mL/min).

Results and Discussion

Reduction of vitamin B₁₂ by Ti(III) citrate

After addition of the reductant, the vitamin B₁₂ solutions turned blue. Figure 29 shows the spectral changes at five minute intervals for the reduction of 5×10^{-5} M vitamin B₁₂ by 8×10^{-3} M Ti(III) citrate. The solution, originally red, gradually turned blue through an intermediate amber color. The visible spectra of the three stages corresponded to published spectra of vitamin B₁₂, vitamin B_{12r}, and vitamin B_{12s}, respectively (Beaven and Johhson, 1955). Blue colored solutions were therefore considered to be positive indication of the presence of fully-reduced vitamin B_{12s}. The solutions in the ampoules prepared as described above all turned a deep blue color on addition of reductant, but spectra were not taken because the ten-fold greater vitamin B₁₂ concentration produced a color intensity that exceeded the sensitivity limits of the spectrophotometer.

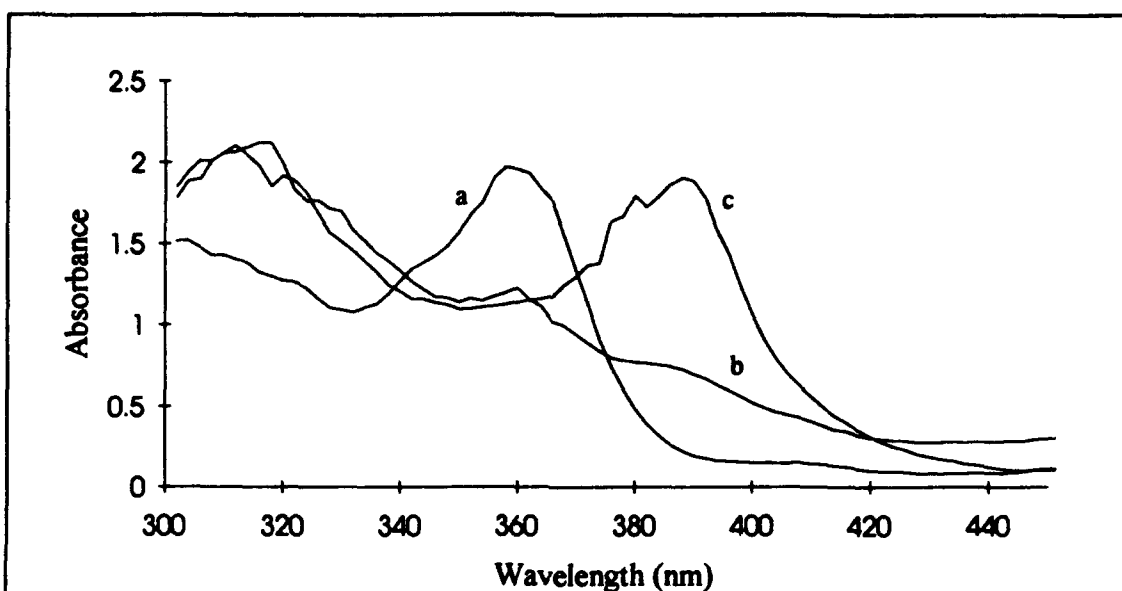


Figure 29. Sequential reduction of vitamin B₁₂ by Ti(III) citrate. The visible spectrum of a 5×10^{-5} M vitamin B₁₂ and 8×10^{-3} M Ti(III) citrate solution was measured at five minute intervals. Vitamin B₁₂ (cyanocob(III)alamin, line a) changes to vitamin B_{12r} (cob(II)alamin, line b), then to vitamin B_{12s} (cob(I)alamin, line c). No further change in the spectrum was observed after ten minutes. The spectra match published spectra of the three oxidation states of vitamin B₁₂ (Beaven and Johnson, 1955).

Reductive dechlorination of chlorophenols

PCP was completely degraded to a mixture of 2,3,4,6-TeCP and 2,3,5,6-TeCP, which were subsequently degraded to a mixture of 2,3,6-TCP and 2,4,6-TCP (Figure 30). Eighty-eight percent of the PCP was degraded within 24 hours, and 96% by 5 days. The molar recovery of the products was 100%. The Ti(III) control reaction for this experiment was conducted with an elevated concentration of Ti(III) citrate and no vitamin B₁₂, and is presented in Figure 30.

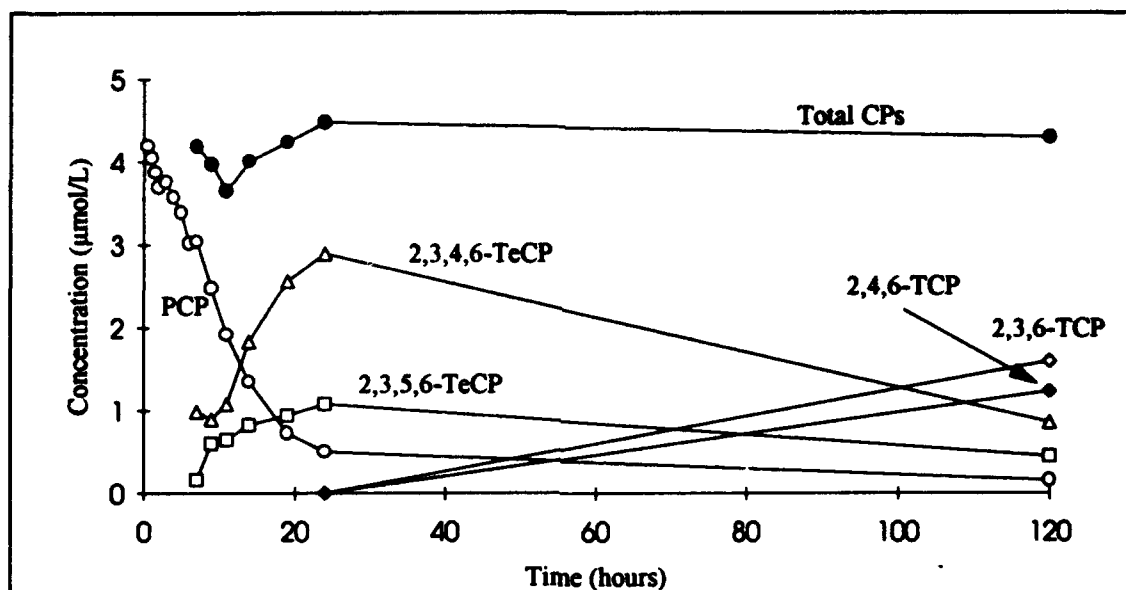


Figure 30. Reaction progress data typical of PCP reductive dechlorination by vitamin B₁₂. Initial concentrations were 4.3×10^{-6} M PCP, 4.8×10^{-4} M vitamin B₁₂ and 0.005 M Ti(III) citrate. Small amounts of TeCPs prior to seven hours were not quantifiable. The reaction was conducted in a TCR.

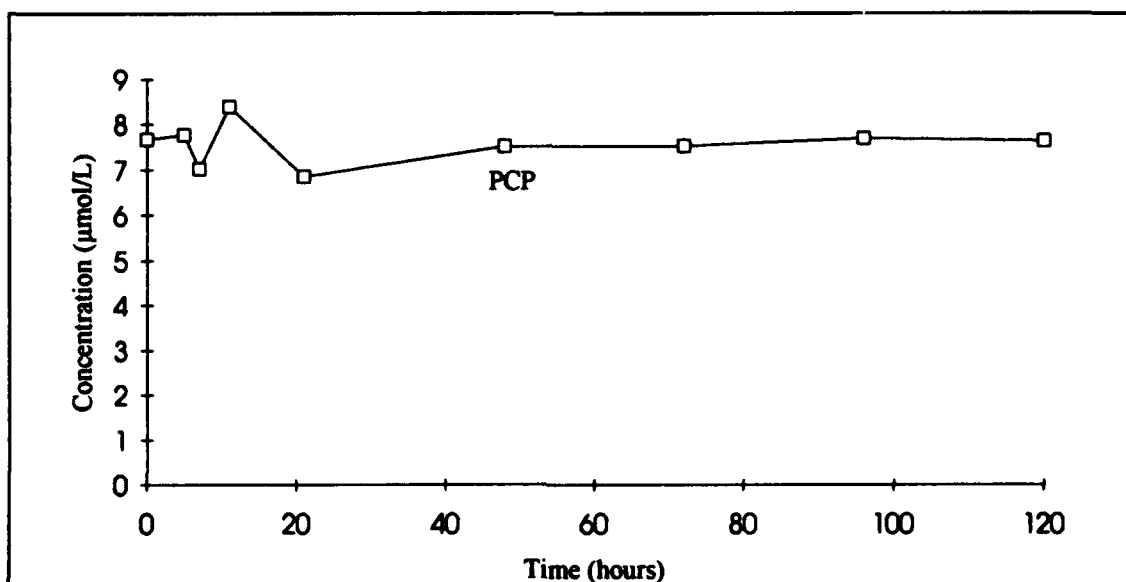


Figure 31. Control reaction for Figure 30. Initial concentrations were 7.7×10^{-6} M PCP and 0.023 M Ti(III) citrate. The reaction was conducted in a TCR.

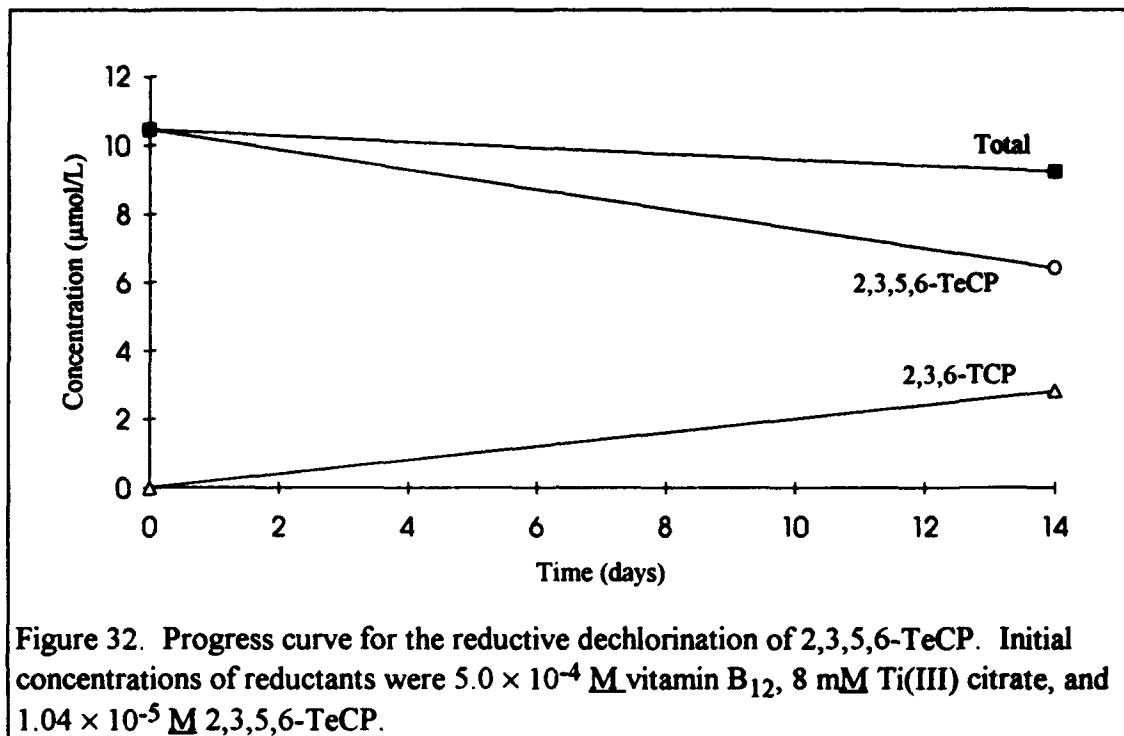
All of the CPs tested were dechlorinated to some extent by vitamin B₁₂. Figures 32 through 39 show progress curves for the reductive dechlorination of the CPs, and Table 5 summarizes the results of all of the dechlorination reactions. The Ti(III) citrate controls shown are for reactions with the chlorophenol and 8 mM Ti(III) citrate with no vitamin B₁₂ present.

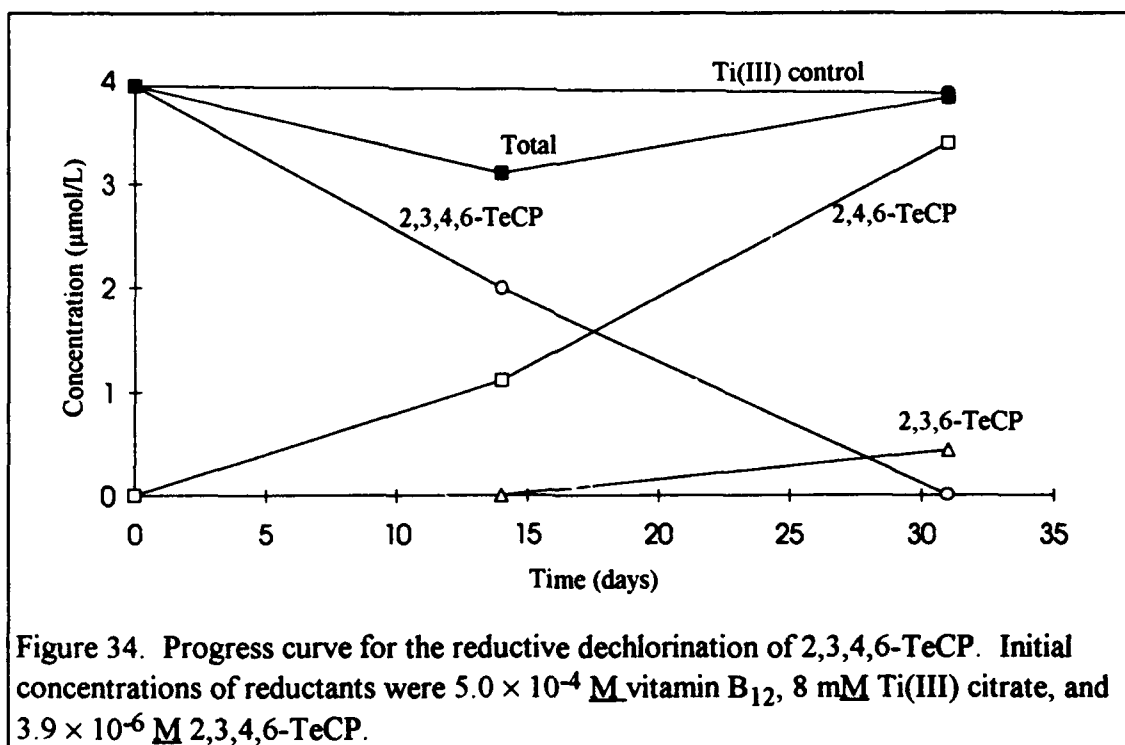
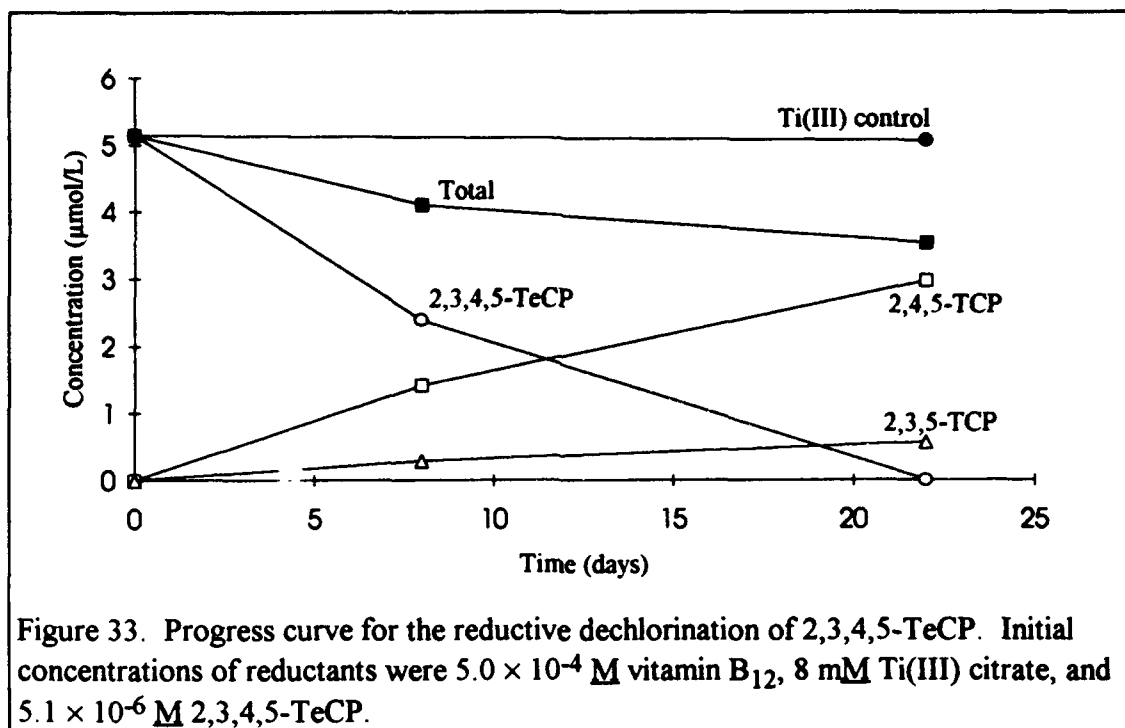
Mass balances for the reactions are all between 57 and 104%. The inability to account for the initial CP mass may indicate that additional transformation products are produced at concentrations below the detection limit of the analytical system. For example, the mass balance for 2,3,4,6-TeCP at day 14 is only 79%, but 2,3,6-TCP was detected below quantification limits. With the accumulation of the trichlorophenols the mass balance at day 31 was 97%.

The chlorines *ortho* to the hydroxyl were least likely to be the target of reductive dechlorination by vitamin B₁₂. No 2,3,4,5-TeCP was detected from the reductive dechlorination of PCP, nor were any 2 chlorines removed from any of the TeCPs. Chlorines adjacent to other chlorines were more likely to be removed than alternative chlorines on the same molecule, excluding chlorines *ortho* to the hydroxyl group. 2,3,4,6-TeCP yielded primarily the 3-dechloro product, 2,4,6-TCP. 2,3,5-TCP yielded predominantly 2,5-DCP, while only 6% of the product was 2,3-DCP. 2,3,4-TCP yielded almost exclusively 2,4-DCP, the 3-chlorine being *ortho* to two other chlorines.

A notable exception to this trend is 3,4,5-TCP, which yielded 72% of the *m*-dechloro product 3,4-DCP. This is in approximate proportion to the isomeric abundance of the chlorines, the 3 and 5 positions being equivalent. The product of reductive dechlorination of 2,4,5-TCP could not be conclusively identified, because 2,4-DCP and 2,5-DCP coelute in the chromatographic method used in this study. However, no 3,4-DCP was detected from this substrate, in keeping with the overall recalcitrance of the 2-chlorine. The only CP for which removal of the chlorine *ortho* to

the hydroxyl was observed was 2,4,6-TCP, a molecule on which no chlorines are *ortho* to any other.





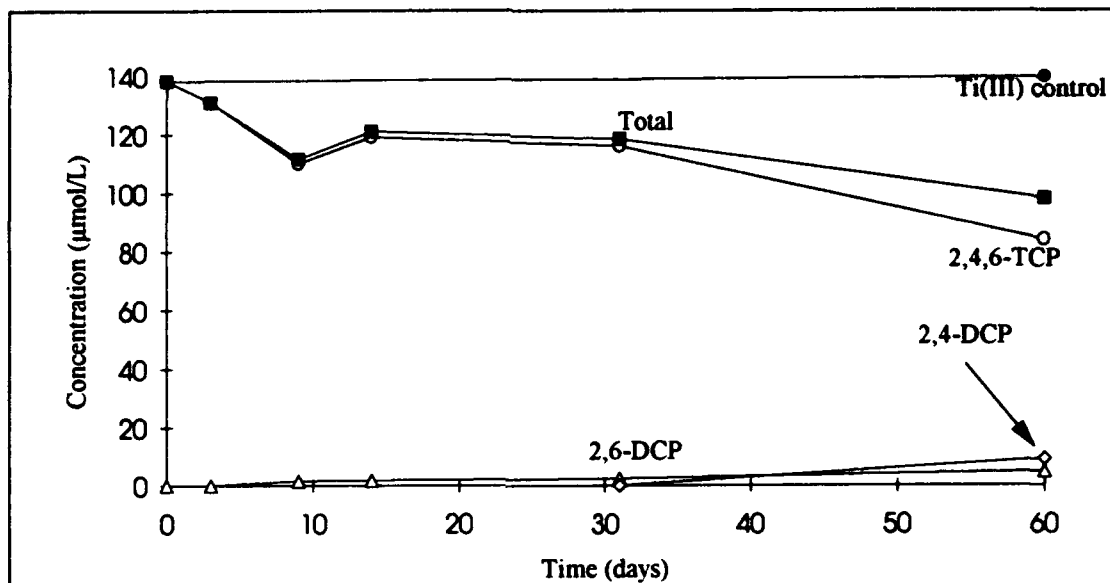


Figure 35. Progress curve for the reductive dechlorination of 2,4,6-TCP. Initial concentrations of reductants were 5.0×10^{-4} M vitamin B₁₂, 8 mM Ti(III) citrate, and 1.4×10^{-4} M 2,4,6-TCP.

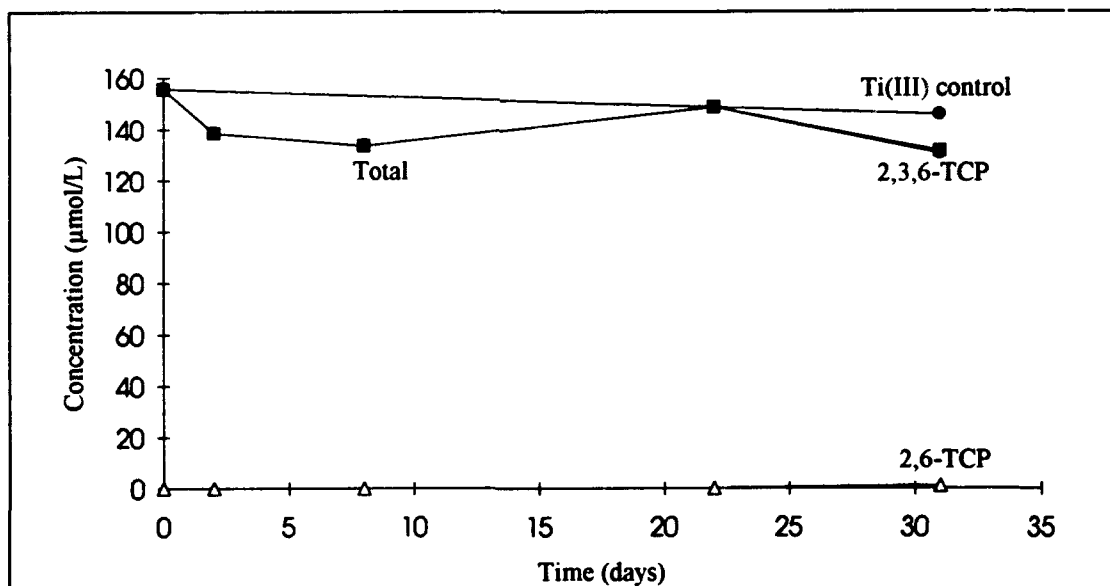
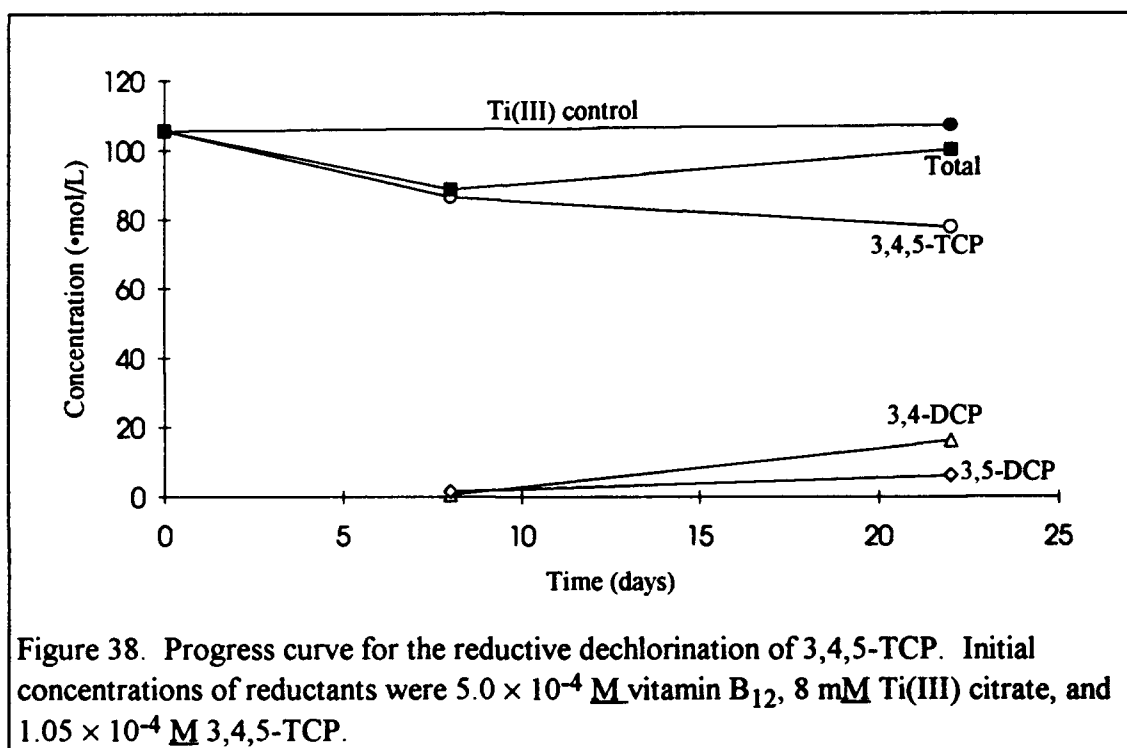
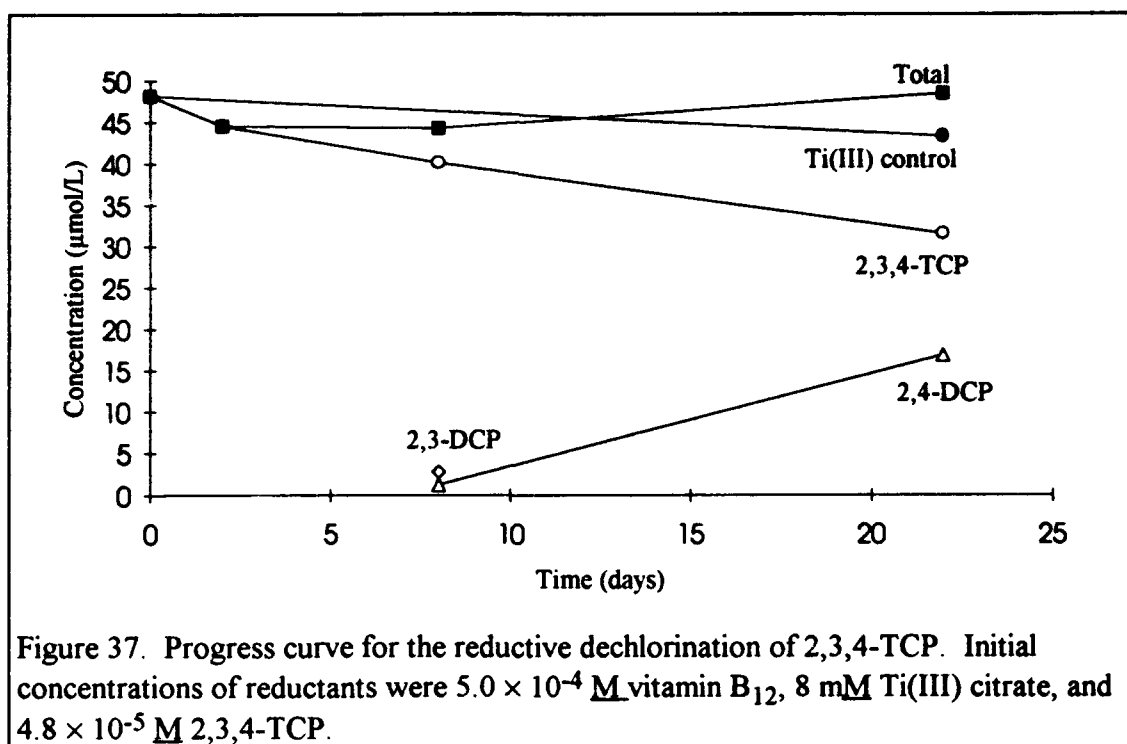


Figure 36. Progress curve for the reductive dechlorination of 2,3,6-TCP. Initial concentrations of reductants were 5.0×10^{-4} M vitamin B₁₂, 8 mM Ti(III) citrate, and 1.56×10^{-4} M 2,3,6-TCP.



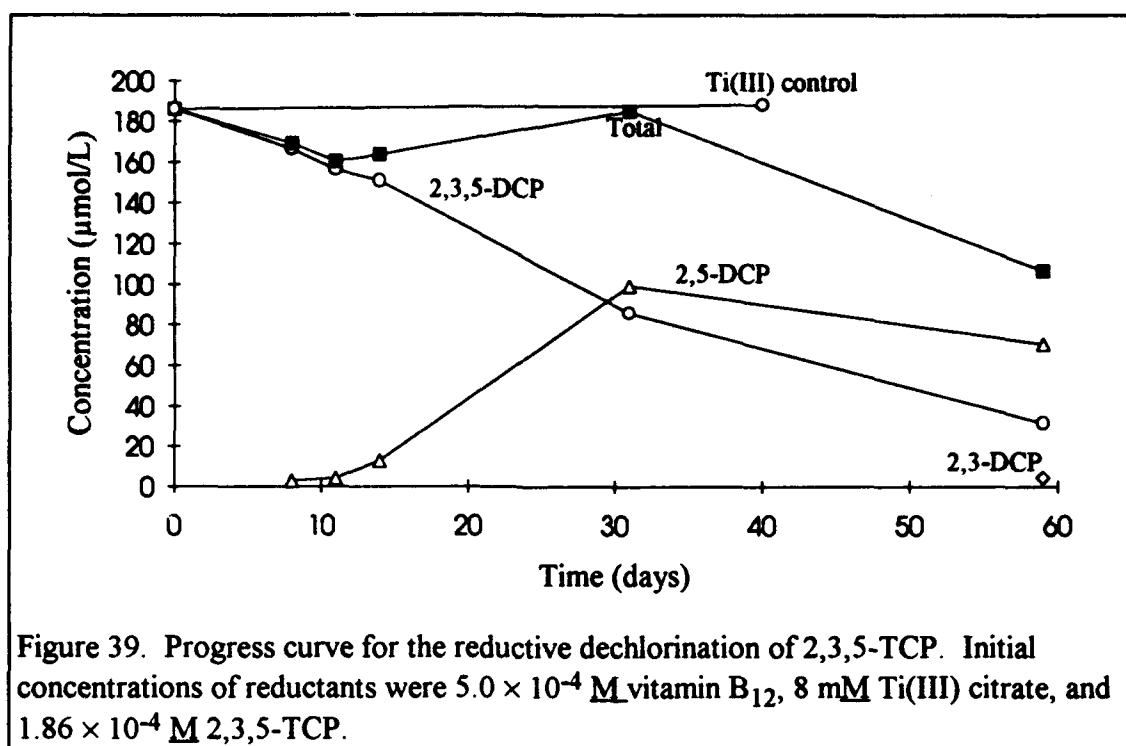


Table 5. Summary of reductive dechlorination of CPs by vitamin B₁₂s. Vitamin B₁₂ concentration was 5×10^{-4} M and Ti(III) citrate 8×10^{-3} M. PCP reductive dechlorination was conducted in a TCR at room temperature. For all other CPs, solutions of reduced vitamin B₁₂ and CP were sealed in glass ampoules and placed in a dark incubator at 27°C to react.

Substrate	Percent conversion	Products (% of products)	Percent recovery ¹	Time allowed for conversion (days)
PCP	88	2,3,4,6-TeCP (73) 2,3,5,6-TeCP (27)	104	1
2,3,4,5-TeCP	100	2,4,5-TCP (84) 2,3,5-TCP (16)	68	22
2,3,4,6-TeCP	100	2,4,6-TCP (88) 2,3,6-TCP (12)	97	31
2,3,5,6-TeCP	37	2,3,6-TCP (100)	88	14
2,3,4-TCP	34	2,4-DCP (100) 2,3-DCP (transient)	99	22
2,3,5-TCP	83	2,5-DCP (94) 2,3-DCP (6)	57	59
2,3,6-TCP	14	2,6-DCP (100)	86	31
2,4,5-TCP	37	2,4-DCP or 2,5-DCP ²	83	31
2,4,6-TCP	39	2,6-DCP (34) 2,4-DCP (65)	71	60
3,4,5-TCP	26	3,4-DCP (72) 3,5-DCP (27)	95	22

¹Sum of moles of all products recovered divided by moles of starting material $\times 100$.

²The dechlorination product for 2,4,5-TCP could not be determined with certainty because 2,4- and 2,5-DCP coelute with the chromatographic regimen used to assay the chlorophenols. No 3,4-DCP was detected.

No reductive dechlorination of any of the substrates was observed with extended contact with Ti(III) citrate alone or with unreduced vitamin B₁₂ alone.

Based upon the observed regiospecificity of the reductive dechlorination of the chlorophenols by vitamin B₁₂, and upon the known chemistry of vitamin B₁₂ and of aryl halides, I propose that vitamin B₁₂s reacts with the chlorophenols by an addition-elimination mechanism (Figure 40). Vitamin B₁₂s, or cob(I)alamin (Cbl(I)), is one of the most powerful nucleophiles known (Schrauzer, 1968). It would be expected to react with aryl halides by nucleophilic aromatic substitution, replacing the chlorine on the ring by an addition-elimination process. The addition step of this process results in a metastable anionic intermediate known as a Meisenheimer, or σ -complex, in which the cobalt atom of the cobalamin is σ -bonded to a ring carbon. The σ -complex is stabilized by electron-withdrawing groups, such as halogens, *ortho* or *para* to the site of attack (McMurry, 1992). In every case, except 2,4,6-TCP, the preferred site of reductive dechlorination is *ortho* or *para* to one or more additional chlorines, but not *ortho* to the hydroxyl (or oxyanion) group. The electron-donating hydroxyl group deactivates positions *ortho* to it to nucleophilic attack.

In the second step of the proposed mechanism, a chloride is eliminated to form an arylcobalamin. Aryl cobalamins are likely to be very unstable. An extensive literature search has failed to provide any references to arylcobalamins, save for a single report of an unsuccessful attempt to make phenyl cobalamin from bromobenzene (Smith et al., 1963). Aryl derivatives of the cobalt chelates bis(diethylphenylphosphine)cobalt (Chatt and Shaw, 1961) and bis(salicylaldehyde)ethylenediiminecobalt [Co(salen)] (Costa et al., 1967) have been prepared by reaction of Grignard reagents in tetrahydrofuran.

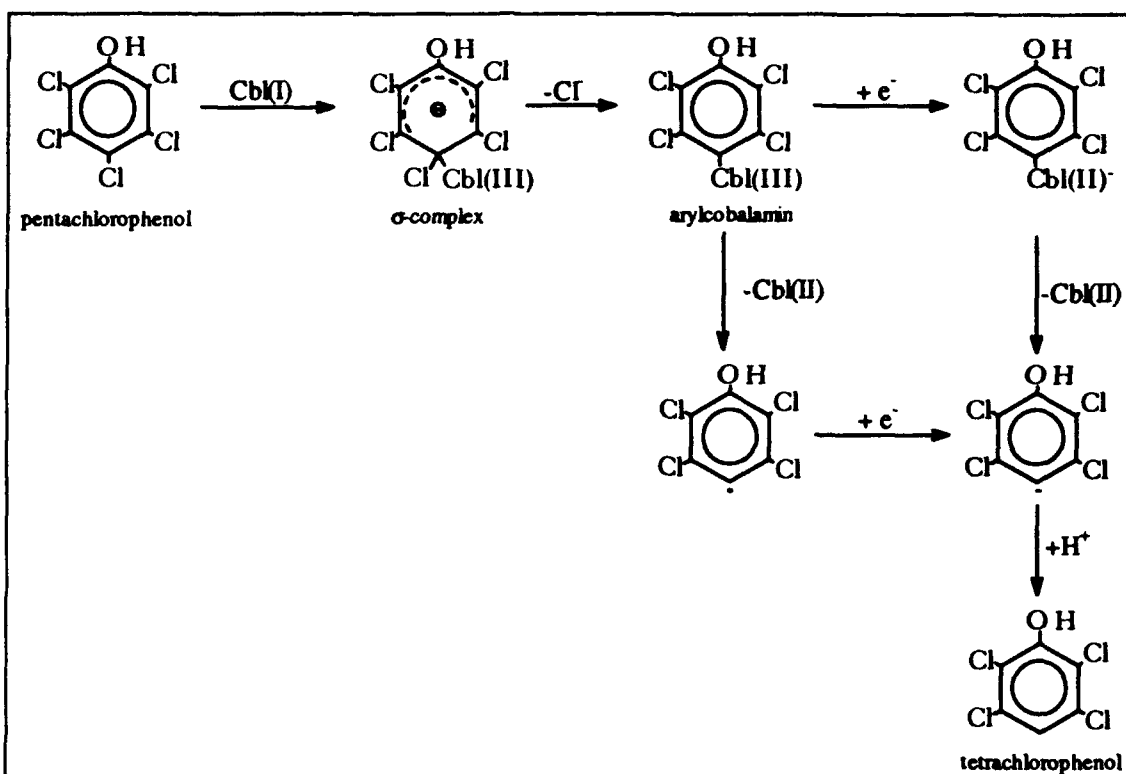


Figure 40. Proposed addition-elimination mechanism for the reductive dechlorination of CPs by vitamin B₁₂s. Vitamin B₁₂s, here abbreviated Cbl(I) for cob(I)alamin, is a very strong nucleophile and attacks at a chlorinated carbon to form a σ -bond with the CP, creating a stable anionic intermediate known as a σ -complex. Cbl(III) indicates that the cobalamin has donated two electrons to the new bond, the Co center being consequently oxidized to the +3 formal valence state. An arylcobalamin is formed upon expulsion of a chloride anion. The Co-C bond can either cleave spontaneously to cob(II)alamin and aryl free radical, which is then reduced to aryl anion, or reduction can occur prior to bond cleavage. The aryl anion abstracts a proton from the reaction milieu to generate the reductively dechlorinated product.

The next step in the proposed mechanism is cleavage of the cobalt-carbon bond. Organocobalamins can cleave spontaneously to form the organic free radical and cob(II)alamin, a process catalyzed by exposure to light or heat (Schrauzer et al., 1968). The organocobalamin may be further reduced by one equivalent and then cleave heterolytically to form the organic anion and vitamin B_{12r} (Costa et al., 1971, 1974; Schrauzer et al., 1972), or homolytically to the organic free radical and vitamin B_{12s} (Hill et al., 1971; Costa et al., 1974).

Secondary cobalamins were long thought to be too unstable to be isolated because of steric restrictions imposed by the corrin ring. Schrauzer and coworkers found that the ring could undergo conformational distortion to accommodate the bulky substituent (Grate and Schrauzer, 1979; Schrauzer and Grate, 1981). In this distorted configuration, the benzimidazole group of the cobalamin is not attached to the β -position of the cobalt; the cobalamin is said to be in its "base off" form. The affinity of the benzimidazole group for the δ^+ charged Co(III) ion is sufficient to promote Co-C bond cleavage (Grate and Schrauzer, 1979).

Nucleophilic attack would be expected to occur at the most electrophilic position on the ring. Cozza and Woods (1992) calculated carbon-chlorine bond electron densities for the unionized chlorophenols using a modified neglect of differential overlap (MNDO) method. Table 6 shows the bond electron densities for each CP and the observed reductive dechlorination product. The optimization procedure selected a spatial orientation for the hydroxyl hydrogen which resulted in asymmetry of charges, even for seemingly symmetrical molecules like PCP and 2,4,6-TCP. With the exception of 2,3,4,5-TeCP, the position of most positive (or least negative) charge corresponds with the reductive dechlorination pattern observed. If 2,3,4,5-TeCP followed the pattern, 2,3,4-TCP would be expected as a product, but none was observed.

Table 6. Calculated net chlorine-carbon bond charge¹ and position of vitamin B₁₂s reductive dechlorination of chlorophenols. In each case except one, the most positive (or least negative) position is subject to attack. The exception is 2,3,4,5-TeCP, where reductive dechlorination at the most positive position is not observed.

Substrate	Chlorine-carbon bond charge ²	Position dechlorinated by vitamin B ₁₂ s
PCP	C2(-0.060), C3(+0.035), C4(-0.060), C5(+0.044) , C6(-0.144)	<i>m, p</i>
2,3,4,5-TeCP	C2(-0.146), C3(-0.005), C4(-0.063), C5(+0.029)	<i>m (3), p</i> (no 2,3,4-TCP)
2,3,4,6-TeCP	C2(-0.072), C3(+0.028), C4(-0.093), C6(-0.174)	<i>m</i>
2,3,5,6-TeCP	C2(-0.148), C3(+0.006), C5(-0.002), C6(-0.065)	<i>m</i>
2,3,6-TCP	C2(-0.078), C3(-0.012) , C6(-0.182)	<i>m</i>
2,4,6-TCP	C2(-0.106) , C4(-0.130), C6(-0.187)	<i>o, p</i>
2,3,5-TCP	C2(-0.151), C3(-0.013) , C5(-0.044)	<i>m</i>
3,4,5-TCP	C3(-0.021), C4(-0.068), C5(-0.014)	<i>p, m</i>
2,3,4-TCP	C2(-0.160), C3(+0.018), C4(-0.099)	<i>m</i>
2,4,5-TCP	C2(-0.180), C4(-0.097), C5(-0.014)	<i>m or p</i>
¹ Cozza and Woods, 1992		
² Most positive or least negative in boldface.		

The position of initial nucleophilic attack is not the only factor operating to determine regiospecificity. Dolfing and Harrison (1992) have calculated the Gibbs free energies of formation for many halogenated aromatic compounds, and used these values to determine the free energy of reductive dehalogenation. The products of reductive dechlorination correlate very well with the ΔG° values (Table 7). In most cases, the most energetically favorable products are the same as those observed. The correlation is not strictly observed, however. For instance, the second most energetically favorable product of 2,3,5-TCP reductive dechlorination, 3,5-DCP, was not observed, while the first and third in the series, 2,5- and 2,3-DCP, were. The 2-chlorine seems especially recalcitrant. The major product was 2,5-DCP, the most energetically favorable product. Only a small amount of 2,3-DCP was detected. It is possible that some 3,5-DCP was produced but not detected, but most likely the

destabilizing effect of the *ortho* hydroxyl on the σ -complex precludes dechlorination at the 2 carbon.

The exception to the recalcitrance of the 2-chlorine to reductive elimination is 2,4,6-TCP. This molecule has no chlorines *ortho* or *para* to any other, and formation of a σ -complex at the 2 position should be unstable due to the proximity of the hydroxyl group. Nucleophilic attack at a protonated aryl carbon can occur when electron-withdrawing groups occupy a 1,3,5 pattern on the ring (Servis, 1965, 1967). A σ -complex of diethylamine and 1,3,5-trinitrobenzene can be formed in this fashion (Servis, 1967). Reductive elimination of the β -chlorine from the cyclopentadienyl ligand to reform the arene could occur in a manner reminiscent of the formation of alkenes from 1,2-dihalogenated alkanes (Shanke and Wackett, 1992).

Table 7. Gibbs free energies of reaction for reductive dechlorination of chlorinated phenols¹ and the products of reductive dechlorination by vitamin B₁₂s.

Substrate	Product	ΔG° kJ/reaction	Observed products
PCP	2,3,4,6-TeCP	-167.8	yes
	2,3,5,6-TeCP	-166.0	yes
	2,3,4,5-TeCP	-156.9	no
2,3,4,5-TeCP	2,3,5-TCP	-154.0	yes
	2,4,5-TCP	-153.6	yes
	2,3,4-TCP	-143.0	no
	3,4,5-TCP	-141.0	no
2,3,4,6-TeCP	2,4,6-TCP	-157.1	yes
	2,3,6-TCP	-151.1	yes
	2,4,5-TCP	-142.8	no
	2,3,4-TCP	-132.2	no
2,3,5,6-TeCP	2,3,6-TCP	-152.9	yes
	2,3,5-TCP	-144.9	no
2,3,4-TCP	2,4-DCP	-166.4	yes
	2,3-DCP	-158.3	yes
	3,4-DCP	-152.9	yes
2,3,5-TCP	2,5-DCP	-157.6	yes
	3,5-DCP	-155.7	no
	2,3-DCP	-147.3	yes
2,3,6-TCP	2,6-DCP	-151.9	yes
	2,5-DCP	-149.6	no
	2,3-DCP	-139.3	no
2,4,5-TCP	2,5-DCP	-157.9	2
	2,4-DCP	-155.7	2
	3,4-DCP	-142.3	no
2,4,6-TCP	2,6-DCP	-146.0	yes
	2,4-DCP	-141.5	yes
3,4,5-TCP	3,5-DCP	-156.0	yes
	3,4-DCP	-142.3	yes

¹ Dolfing and Harrison (1992), calculated for pH 7.0, 1 molal concentration, and H₂ as electron donor.

² Analytical method could not discern between 2,4- and 2,5-DCP. One or both products were observed.

The pattern of reductive dechlorination by vitamin B_{12s} for all of the tri- to pentachlorophenols, constructed from these observations, is shown in Figure 41. This dechlorination pattern most resembles that observed with anaerobic consortia acclimated to 3-CP (Mikesell and Boyd, 1986), which reductively dechlorinated PCP to a mixture of 2,3,4,6-TeCP and 2,4,6-TCP. However, this population of bacteria did not produce products of dechlorination at the 4 position, which vitamin B_{12s} does. Anaerobic consortia acclimated to 4-CP (Mikesell and Boyd, 1986) produced 2,3,5,6-TeCP, but no 2,3,4,6-TeCP, followed by removal of an *ortho* chlorine to produce 2,3,5-TCP, which was not detected in the present study.

Although the present study was designed to determine the regiospecificity of vitamin B₁₂s reductive dechlorination, a general idea of the relative rates of reductive dechlorination may be observed from these results. The relative rate of reductive dechlorination was directly proportional to the degree of dechlorination. Figure 42 shows the progress of reductive dechlorination for all the CPs, normalized to their initial concentrations. PCP reductive dechlorination is clearly faster than that for the other CPs. The progress curves for the TeCPs lie between those for PCP and the TCPs.

Vogel et al. (1987) suggested that the rate of reductive dechlorination of chlorinated aliphatic compounds should be proportional to the standard reduction potential of the compound. Gantzer and Wackett (1991) observed a linear relationship between the logarithm of the pseudo-first order rate constant for reductive dechlorination of chloroethylenes by vitamin B₁₂s and the half-reaction reduction potentials of the substrates. If first order rate constants are estimated from the data presented in Figure 42, then a similar relationship emerges. Dolfing and Harrison (1992) calculated the redox potentials for the reductive dechlorination of the chlorophenols (Table 7) by H₂. Figure 43 shows the estimated rate constants plotted as a function of these redox potential for the conversion of the substrate to the most prevalent product (Table 5). The overall trend is consistent with that observed for the chlorinated ethylenes.

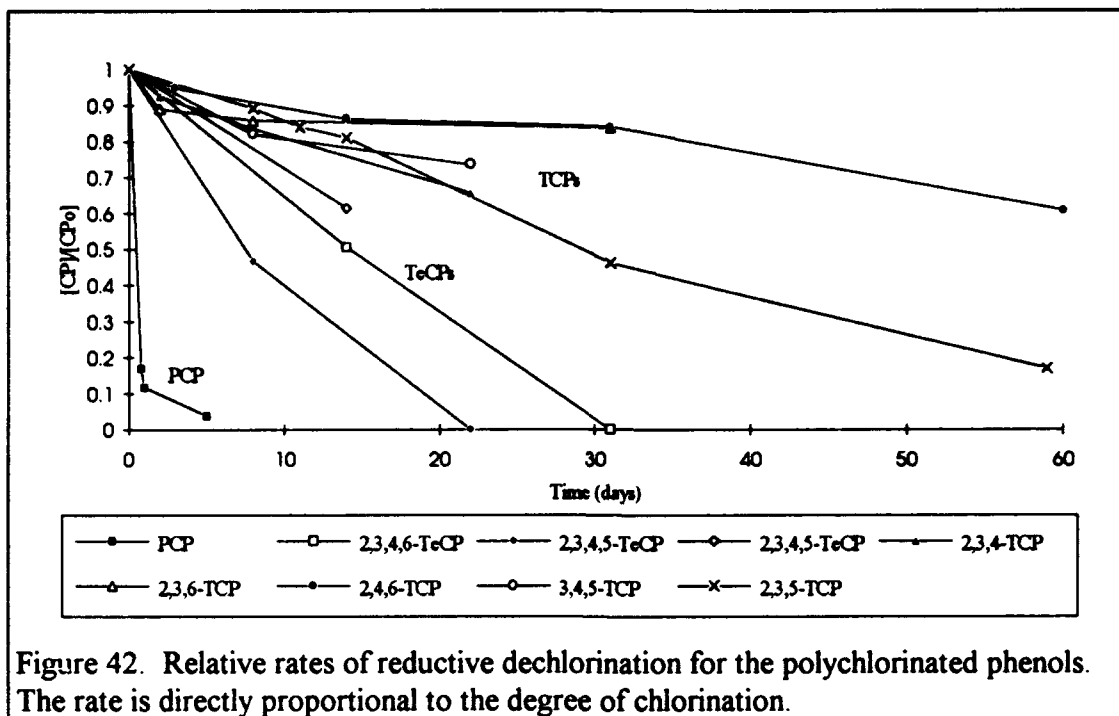


Figure 42. Relative rates of reductive dechlorination for the polychlorinated phenols. The rate is directly proportional to the degree of chlorination.

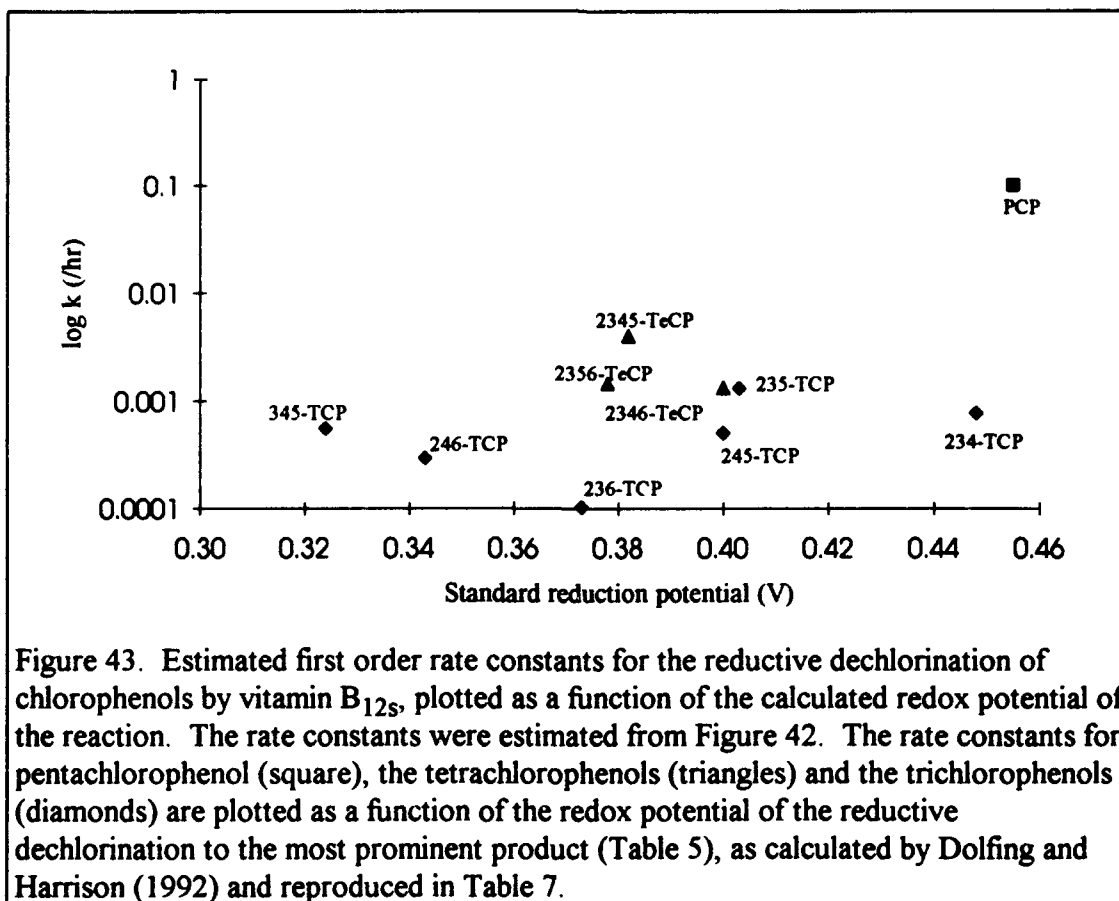


Figure 43. Estimated first order rate constants for the reductive dechlorination of chlorophenols by vitamin B_{12} , plotted as a function of the calculated redox potential of the reaction. The rate constants were estimated from Figure 42. The rate constants for pentachlorophenol (square), the tetrachlorophenols (triangles) and the trichlorophenols (diamonds) are plotted as a function of the redox potential of the reductive dechlorination to the most prominent product (Table 5), as calculated by Dolfig and Harrison (1992) and reproduced in Table 7.

Recovery of substrate and products as the reactions progressed was generally less than the starting material, suggesting that further degradation or conversion to unanticipated products was occurring. The ECD detector response decreases as the degree of chlorination decreases, so that small quantities of further reduction products may easily be missed. Krone et al. (1989b) could not account for all of the starting material in their studies of the reductive dechlorination of chlorinated methanes by vitamin B₁₂s. They suggested that condensation of free radical intermediates to non-volatile products was responsible for their observations.

Reduced vitamin B₁₂ is potentially capable of contributing to the transformation of CPs in the environment, but the pathways established in this study suggest that the simple presence of reduced cobalamin in the environment cannot by itself explain the biological reductive dechlorination of PCP. The regiospecificity of dechlorination exhibited by vitamin B₁₂s is different from that of both unacclimated and acclimated anaerobic consortia. While it most resembles the pathway exhibited by anaerobic sludge acclimated to 3-MCP, the recalcitrance of the *ortho* chlorine to removal by vitamin B₁₂s cannot be reconciled with the eagerness with which acclimated and unacclimated anaerobic consortia alike remove the *ortho* chlorine.

Corrinoids in nature function as cofactors together with apoenzymes which direct their natural chemistry toward specific purposes (Pratt, 1972). While corrinoids generally can react as methyl or hydride transfer agents, it is the intact enzymatic apparatus that directs and controls the ultimate outcome of the individual chemical process. It is not unreasonable to expect that such would be the case for reductive dechlorination of organic halides.

Conclusions

Vitamin B_{12s} catalyzes the reductive dechlorination of chlorophenols preferentially at positions adjacent to other chlorinated carbons. The 2 position is resistant to reductive dechlorination. The regiospecificity is consistent with an addition-elimination mechanism of attack by the cobalamin, with substitution of the chlorine by nucleophilic aromatic substitution. The rate of reductive dechlorination is proportional to the degree of chlorination, and roughly proportional to the standard reduction potential of the reaction. Vitamin B₁₂ reductive dechlorination regiospecificity differs substantially from that exhibited by anaerobic microbial consortia.

Chapter 7

Kinetics of Pentachlorophenol Reductive Dechlorination by Vitamin B₁₂

Abstract

Reductive dechlorination of pentachlorophenol by vitamin B₁₂, reduced to vitamin B₁₂s by Ti(III) citrate, was studied. Illumination of the reaction has little or no effect on the reaction rate. The reaction begins at a slow rate and speeds up as time progresses. The shape of the progress curve can be modeled either as an autocatalytic reaction or as an artifact of the analytical protocol and the addition-elimination reaction mechanism. In either case, decay of intermediates to chlorophenol congeners must occur for the models to describe the reaction. In this study, a large excess of vitamin B₁₂ was used. The extent of reaction, if not the shape of the reaction curve, is adequately described with a simple model of a pseudo-first order reaction in PCP.

Introduction

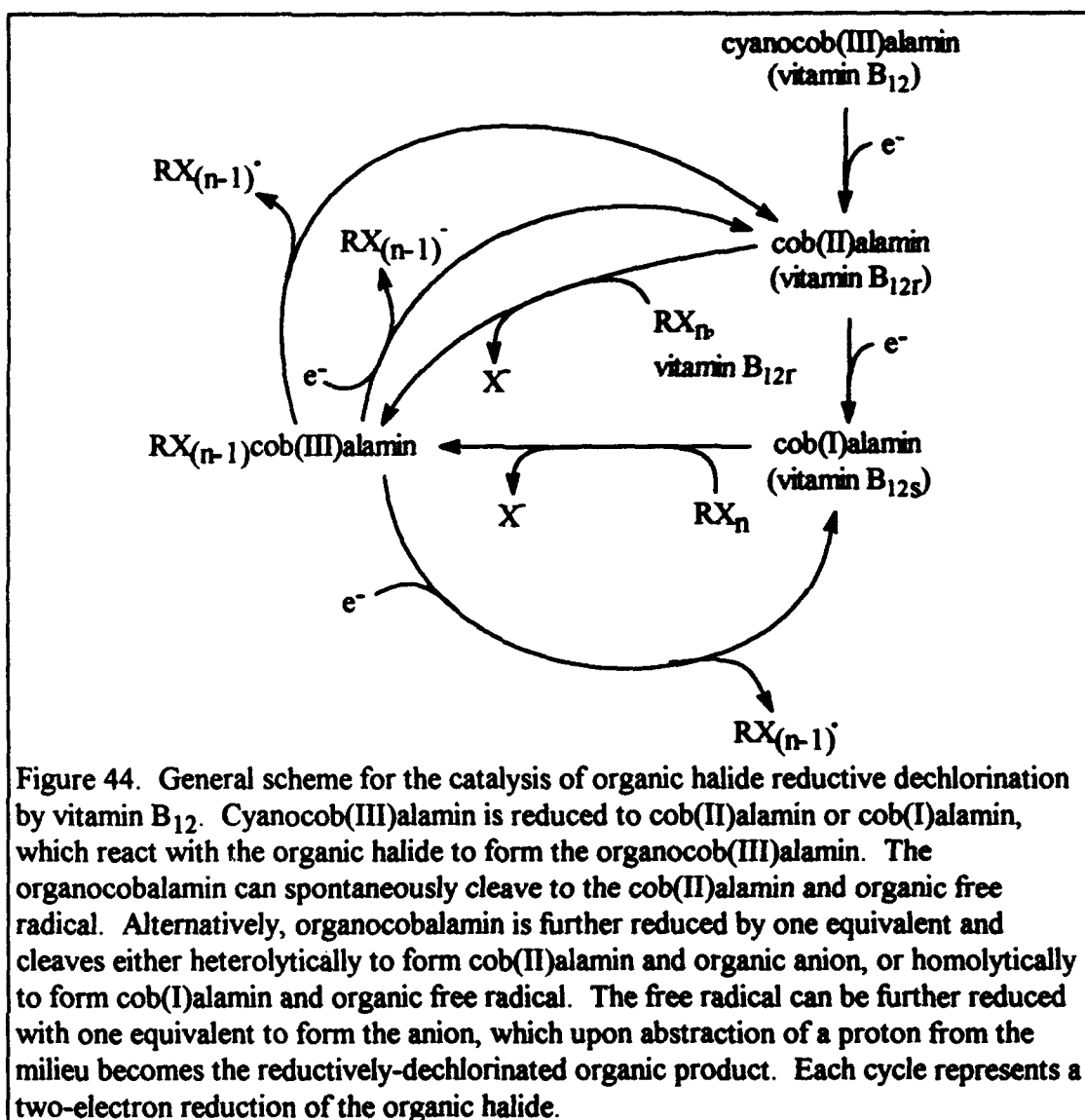
Natural and engineered biological systems can transform environmental pollutants into less noxious agents. A thorough understanding of the biochemical reactions involved in these transformations may lead to improved processes for bioremediation of hazardous waste sites. A transformation of particular interest is reductive dechlorination, as a large number of hazardous substances of environmental concern are chlorinated hydrocarbons.

In reductive dechlorination, the carbon to which a chlorine is attached is reduced while the chlorine is exchanged for a hydrogen. Reductive dechlorination has been observed in soils, sediments, aquifers, and sewage sludge. A variety of chlorinated

hydrocarbons are reductively dechlorinated, including chlorinated methanes, ethanes, and ethenes, aromatic chlorides, and chlorinated pesticides. These have been recently reviewed (Tiedje et al., 1987; Mohn and Tiedje, 1992).

Several naturally-occurring metalloorganic compounds have been shown to catalyze reductive dechlorination, and the implications to natural processes have been recognized (Krone et al., 1989a, b; Gantzer and Wackett, 1991). Carbon tetrachloride, perchloroethylene and their dechlorinated congeners are rapidly reductively dechlorinated by the vitamin B₁₂, with Ti(III) citrate or dithiothreitol as electron donor (Krone et al., 1989a, b; Gantzer and Wackett, 1991).

From the vast literature covering three decades of inquiry into the synthesis, nature and fate of alkyl cobalamins and similar model compounds, a generalized model may be constructed for the catalytic participation of vitamin B₁₂ in reductive dehalogenation. Figure 44 shows the catalytic cycle for the reductive dehalogenation of organic halides by vitamin B₁₂. Vitamin B₁₂ (cyanocob(III)alamin) can be reduced to the Co(II) oxidation state to form vitamin B_{12r}, or to the Co(I) oxidation state to form vitamin B_{12s}, one of the most powerful nucleophiles known (Schrauzer et al., 1965). Vitamin B_{12s} reacts with alkyl halides by an S_N2 mechanism (Schrauzer and Deutsch, 1969), releasing a free halide anion and forming a σ -bond between the cobalt center and the carbon which formerly hosted the released halide. Alternatively, two equivalents of vitamin B_{12r} react with an alkyl halide by a free radical mechanism to form the alkylcobalamin (Halpern and Maher, 1965; Blaser and Halpern, 1980). The fate of the organocobalamin is dependent upon the nature of the organic ligand and environmental conditions.



The organocobalamin can cleave spontaneously to the organic free radical and cob(II)alamin, a process catalyzed by exposure to light or heat (Schrauzer et al., 1968). The organocobalamin may be further reduced by one equivalent and then cleave heterolytically to form the organic anion and vitamin B_{12r} (Costa et al., 1971, 1974; Schrauzer et al., 1972), or homolytically to the organic free radical and vitamin B_{12s} (Hill et al., 1971; Costa et al., 1974).

The kinetics of reductive dechlorination of chlorinated methanes and ethanes are first order in both chlorinated hydrocarbon and vitamin B₁₂ (Gantzer and Wackett,

1991). The rate of reaction decreases by approximately an order of magnitude with each successive loss of a chlorine, commensurate with the change in standard reduction potential of the chlorocarbon substrate. Hill et al. (1971) observed the same rate of methane production in a controlled potential reduction cell from methylcobalamin as from methyl iodide in the presence of vitamin B_{12s}, while Krone et al. (1989b) observed faster evolution of methane from CCl₄ by Ti(III) citrate with methylcobalamin as catalyst than from methylcobalamin alone.

Vitamin B₁₂ has recently been shown to catalyze the reductive dechlorination of the aromatic halides pentachlorophenol (PCP), pentachlorobenzene (Gantzer and Wackett, 1991), hexachlorobenzene and 2,3,4,5,6-pentachlorobiphenyl (Assaf-Anid et al., 1992). We have reported elsewhere (Chapter 6) that vitamin B_{12s} also reductively dechlorinates lesser chlorinated chlorophenol congeners in a pattern that suggests nucleophilic substitution on the ring by the cobalamin as an intermediate step. In the present study, we examine the kinetics of reductive dechlorination by vitamin B_{12s}.

Materials and Methods

All reactions were conducted in 0.66 M Tris (enzyme grade, Life Technologies, Inc., Gaithersburg, MD), pH 8.2. Vitamin B₁₂ was obtained from Sigma Chemical Company (St. Louis, MO). PCP, 99.9% purity, was purchased from Sigma. 2,3,4,6- and 2,3,5,6-tetrachlorophenols (TeCP) were purchased from Ultra Scientific, Inc. (N. Kingston, RI, 95+% purity). All chlorophenols were first dissolved in methanol, then diluted in the Tris buffer for transfer into the reaction mixtures. They were dissolved in methanol for use as chromatography standards. A stock solution of 250 mM Ti(III) citrate was prepared as previously described (Chapter 5). Argon was commercial grade, and was purified by passing through an OMI-1 indicating oxygen scavenger (Supelco, Inc., Bellefonte, PA). The gas train was all of copper or fused silica as described previously (Chapter 5).

Reduction of Vitamin B₁₂ by Ti(III) citrate

A glass spectrophotometer cell was modified by stretching in a flame to introduce a narrow neck, in a manner analogous to the preparation of the two-chambered reactor (TCR) described previously (Chapter 5). This cell was fitted with a rubber septum through which the purging capillaries were introduced. A solution of vitamin B₁₂ was purged in this cell for 15 minutes, after which stock Ti(III) citrate solution was added by syringe to make a final concentration of 8 mM Ti(III) citrate and 5.0×10^{-5} M vitamin B₁₂. Spectral changes during the reduction of the vitamin B₁₂ were recorded with a Hewlett-Packard model 8452A phased array recording spectrophotometer. The cell was mixed with a flea-sized magnetic stir bar throughout the reaction.

Reactions in TCRs

Three mL of a solution of vitamin B₁₂ in Tris buffer was placed in the lower chamber of a purged TCR as described previously (Chapter 5). The vitamin solution

was sparged with argon for 15 min to remove O_2 , and the sparging capillary was raised to purge the upper chamber. Ti(III) citrate was added and the solution was allowed to react until the vitamin B_{12} solution turned blue, indicating reduction to vitamin B_{12s} . PCP in buffer solution was added to begin the reaction. Reactions were conducted at ambient temperature with constant mixing provided by a magnetic stir bar in the reactor. Samples were removed through the exhaust capillary at intervals for analysis of chlorophenol concentrations by gas chromatography. Initial concentrations of reactants varied, and were as described in the results section.

In experiment 306, the TCR was placed directly over the magnetic stirring mechanism. In experiments 314 and 330a, the TCRs were placed slightly outside the center of the stir plate to accommodate other experiments.

Effect of light on reductive dechlorination kinetics

Two TCRs (experiments 330a and b) were prepared side-by side with equal argon purge rates. One reactor (a) was completely covered with aluminum foil. A 15-watt halogen reading lamp was directed on the reactors from a distance of 20 cm. The light intensity was measured to be 950 Lux. Vitamin B_{12} concentration was 9.1×10^{-4} M and initial PCP and Ti(III) citrate concentrations were 9.0×10^{-6} M and 2.3×10^{-2} M, respectively. Samples were taken at intervals to monitor the progress of the reactions.

Reactions in serum vials

Two reactions were conducted in 10-mL serum vials. In the first (experiment 249), the serum vial was capped with a Teflon/rubber septum covered with aluminum foil, and agitated on a laboratory shaker throughout the experiment. This experiment was conducted prior to the development of the TCR, and required initial concentrations of Ti(III) citrate of 0.2 M in order to maintain the vitamin B_{12} as vitamin B_{12s} .

throughout the experiment. The vitamin B₁₂ concentration was 1.0×10^{-3} M and initial PCP and Ti(III) citrate concentrations were 2.2×10^{-6} M and 0.20 M, respectively.

The second serum vial experiment (experiment 330c) was conducted in a 10-mL serum vial capped with a butyl rubber septum and covered with foil. A six mm magnetic stir bar placed in the vial provided mixing. The vial was placed on the stir plate slightly off the center to accommodate other experiments.

Reactions in ampoules

One mL aliquots of a solution containing vitamin B₁₂ and PCP were dispensed to several 2 mL glass ampoules (Wheaton Scientific, Millville, NJ). Each ampoule was in turn purged for five minutes with argon, 100 μ L of stock Ti(III) citrate were added, and the ampoule sealed in a flame. Initial concentrations were 9.1×10^{-4} M vitamin B₁₂, 0.024 M Ti(III) citrate, and 9.0×10^{-6} M PCP. Within five minutes the originally red solution had turned a deep blue, indicating reduction to vitamin B_{12s}. The ampoules were placed in a dark incubator at 27°C. At intervals, an ampoule was sacrificed and the contents assayed for chlorophenols.

Table 8 summarizes the reaction conditions for the seven experiments.

Table 8. Reaction conditions for several investigations of the kinetics of PCP reductive dechlorination by vitamin B₁₂s.

Exp #	Vitamin B ₁₂ (M)	PCP (M)	Ti(III) citrate (M)	Light	T (°C) ¹	Reactor, agitation ²
249	1.0×10^{-3}	2.2×10^{-6}	0.20	dark	25	vial, 2
306	5.7×10^{-4}	3.1×10^{-6}	0.008	room	18	TCR, 2
314	4.8×10^{-4}	4.3×10^{-6}	0.005	room	22	TCR, 1
330a	9.1×10^{-4}	9.0×10^{-6}	0.024	dark	23	TCR, 1
330b	9.1×10^{-4}	9.0×10^{-6}	0.024	lamp	23	TCR, 1
330c	9.1×10^{-4}	9.0×10^{-6}	0.024	dark	27	ampoule, 0
330d	9.1×10^{-4}	9.0×10^{-6}	0.024	dark	23	vial, 0
¹ Room temperature.						
² Agitation level estimated from reaction records. No or little agitation, 0; maximum agitation 2; intermediate agitation 1						

Analytical method

The fate of the chlorophenol reactants was followed by gas chromatography using a modification of a miniaturized version (Perkins, 1992) of the acetylation procedure of Voss et al. (1980). One-hundred μ L samples were mixed in a screw-top culture tube with exactly one mL of a reaction medium containing 43 g/L K₂CO₃ and one mg/L 2,4,6-tribromophenol as internal standard. One-hundred μ L of acetic anhydride was added, the tube was capped with a Teflon-lined cap, and shaken on a wrist-action shaker for 20 minutes. Three mL of hexane were then added, and the tube shaken for an additional 10 minutes. The hexane fraction was transferred to an autosampler vial and capped with a Viton septum and crimp-seal cap. Gas chromatography was performed on the hexane extracts with a Hewlett-Packard model 5890A gas chromatograph equipped with an electron capture detector and fitted with a J&W Scientific DB-5 30 m \times 0.32 mm I.D. column. Injector and detector

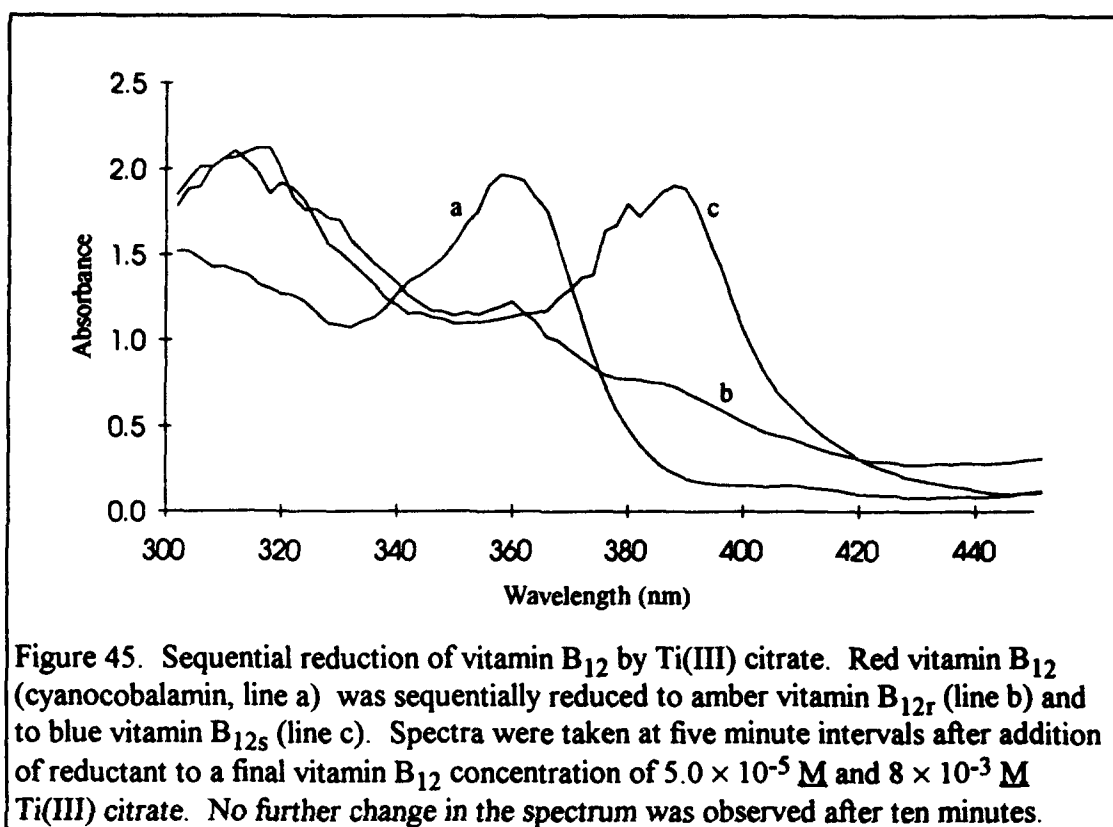
temperatures were 250 and 320°C, respectively. A 1- μ L aliquot was introduced by splitless injection. Initial oven temperature was 45°, which was held for two minutes, followed by a 15°/min ramp to 105° and a 5°/min ramp to 215°, which was held for 5 minutes. The carrier was helium (1 mL/min), and the makeup gas was argon-methane (95%-5%, 60 mL/min).

Results and Discussion

Reduction of Vitamin B₁₂ by Ti(III) citrate

Vitamin B₁₂ is reduced by Ti(III) citrate to the supernucleophile vitamin B_{12s} (cob(I)alamin) (Schrauzer et al., 1968). Figure 45 shows the transition of a solution of 5×10^{-5} M vitamin B₁₂ from the red cyanocobalamin to the blue cob(I)alamin. The color is dependent on the light source. The solution appears blue under reflected fluorescent laboratory lights, but purple when backlit by a halogen incandescent lamp. Under natural sunlight, it is a greenish-gray color, corresponding to earlier published reports (Smith et al., 1963; Beaven and Johnson, 1955). Ti(III) citrate solution is pale blue, which may contribute to the perceived color of reduced vitamin B₁₂ solution.

Comparison of published standard reduction potentials of -0.480 V at pH 7.0 for Ti(III) citrate (Zehnder and Wuhrman, 1976) and -0.61 V for vitamin B_{12s} (Lexa and Saveant, 1983) would suggest that the equilibrium should not favor the formation of vitamin B_{12s}. However, Krone et al., pointed out (1989b) that if the redox couple is considered to be $\text{H}_2\text{O} + \text{Ti}^{3+} = \text{TiO}^{2+} + 2\text{H}^+ + \text{e}^-$, then oxidation of the Ti^{3+} would be favored as the pH increases.



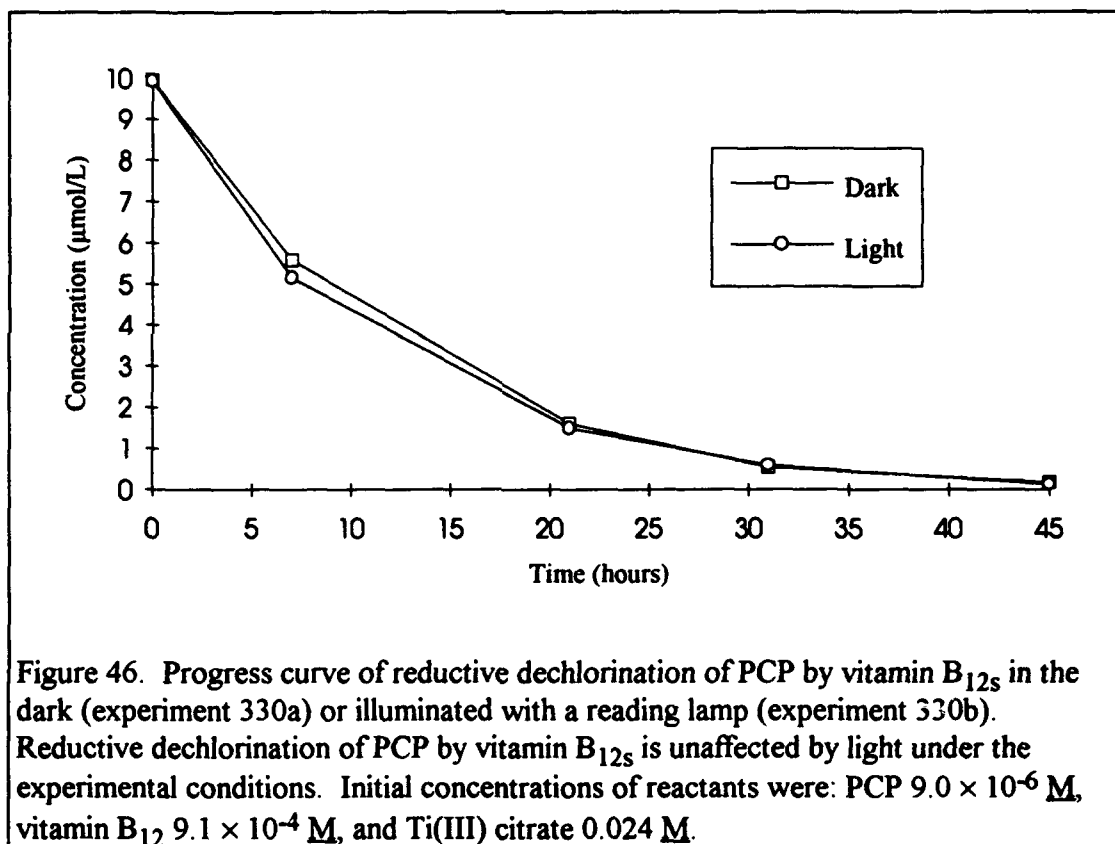
Effect of light on reductive dechlorination kinetics

Two TCRs were operated side by side, one covered with aluminum foil to exclude light and the other continuously exposed to white light at 950 Lux. Figure 46 shows that exposure to light had little effect on the progress of the reaction. In this experiment, the concentration of vitamin B₁₂ exceeded that of PCP by two orders of magnitude, with excess Ti(III) citrate. Under these conditions, the reaction may be reasonably assumed to proceed as pseudo-first order in PCP, the other reactants being consumed in insignificant amounts (Levenspiel, 1972). Under these circumstances, the reaction can be considered to be

$$\frac{d[\text{PCP}]}{dt} = k_1 [\text{B}_{12}] [\text{PCP}] = k_{\text{obs}} [\text{PCP}],$$

where k_{obs} is the observed pseudo-first order rate constant. The data were transformed as is appropriate for first order reactions, and analyzed as such. Figure 47 shows the

plot of the data transformed as the negative logarithm of the ratio of concentrations at time t to the initial concentration. If such a plot is linear, then the reaction follows first order kinetics and the slope is equivalent to the first order rate constant. The data were subjected to multiple linear regression (Appendix D). The rate of reaction was found to be 0.090 hr^{-1} for the reaction in the dark, and 0.093 hr^{-1} for the illuminated reaction. The difference was statistically insignificant ($p = 0.6$). Thereafter, no precautions were taken to shield the reaction from normal room light.



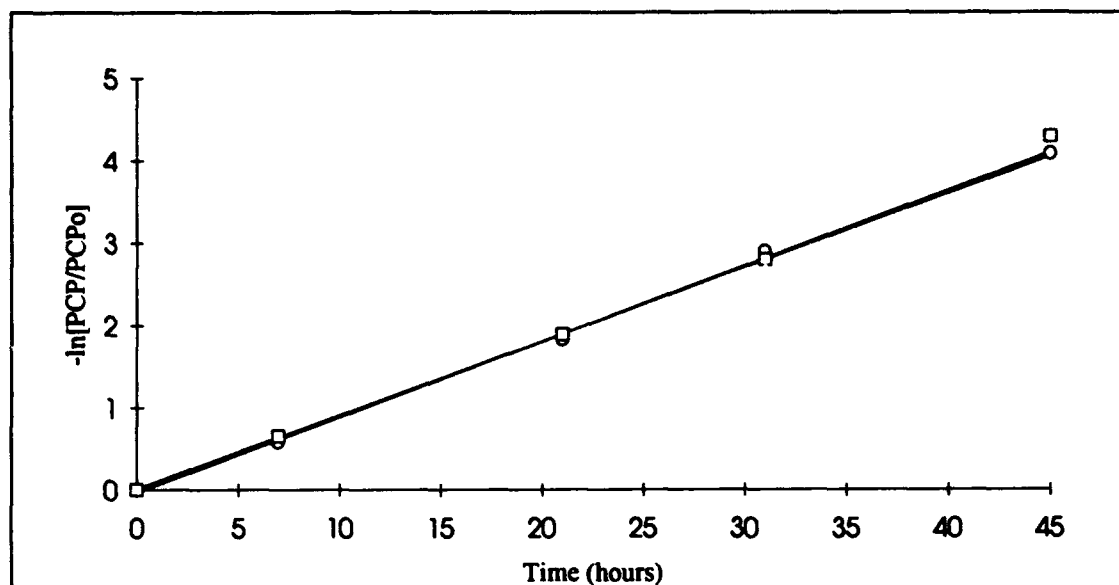


Figure 47. Kinetic data of PCP reductive dechlorination in the dark (circles) or exposed to light (squares), transformed as a pseudo-first order reaction. Initial concentration of reactants was 9.1×10^{-4} M vitamin B₁₂, 9.0×10^{-6} M PCP and 0.024 M Ti(III) citrate. The linear relationship indicates the appropriateness of the pseudo-first order kinetic assumption.

Organocobalamins are known to be sensitive to light, which promotes homolytic bond fission to form cob(II)alamin and organic free radical (Schrauzer et al., 1986). The absence of stimulatory effect of light on the rate of reaction indicates that the rate-limiting step in the reaction is not due to the homolytic cleavage of the carbon-cobalt bond.

Kinetics of reductive dechlorination of PCP by vitamin B₁₂s

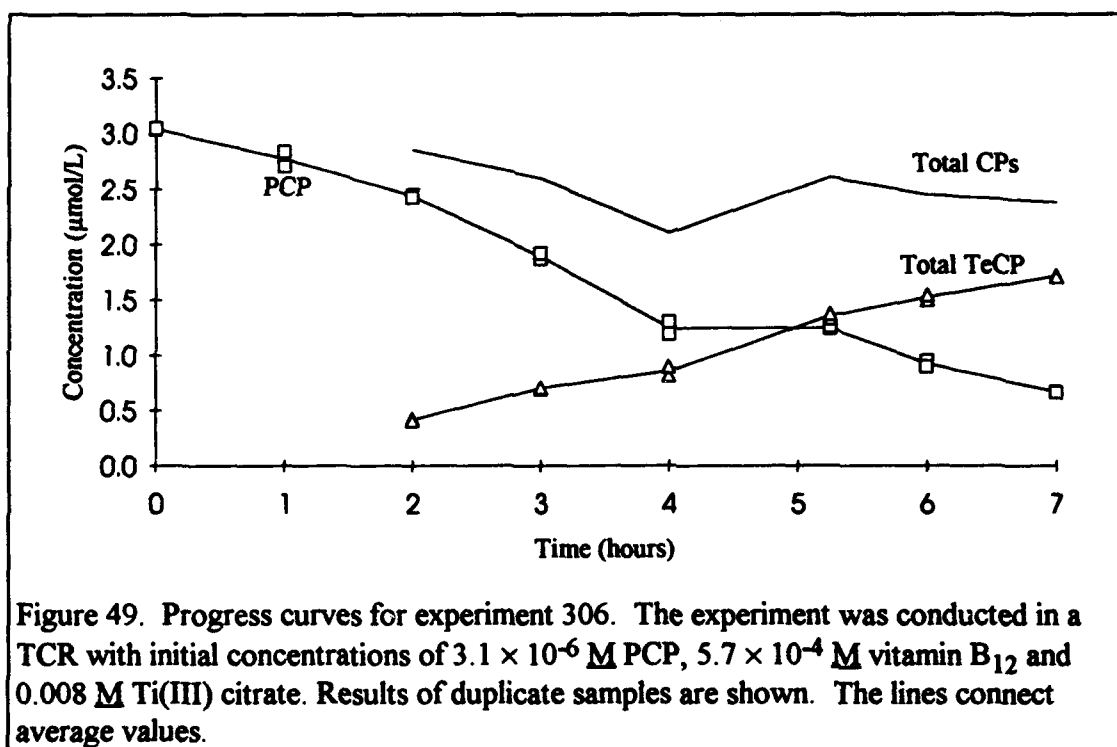
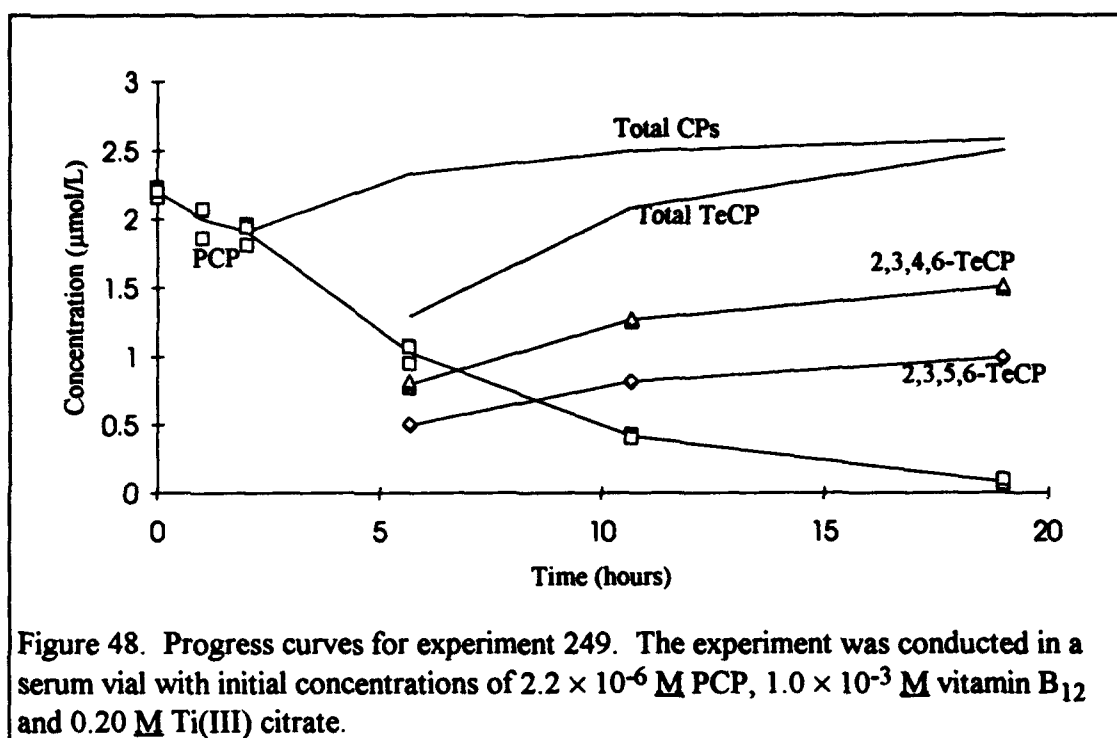
Progress curves for the rest of the reactions are shown in Figures 48 through 51. TeCP levels and mass balances are not shown for the early portions of the experiments because the products, while visible on the chromatograms, were below the quantification limits of the analytical procedure. The data in Figure 48 are the average of triplicate samples, and the data in Figures 49 and 50 are the average of duplicate samples. For all other progress curves, single samples were taken.

First order rate model

Careful examination of the first few hours of experiments 249 (Figure 48), 306 (Figure 49) and 314 (Figure 50) suggests that the first-order rate assumption may not be entirely satisfactory. In these experiments especially, a period of relatively slow PCP disappearance was followed by a period of more rapid decay. On the other hand, all of experiments 330 (Figures 46 and 51) follow classical first order kinetics.

While the PCP concentration profile resembles a first order decay curve, transformation of the data from experiment 314 as a first order reaction yields a non-linear plot (Figure 52). Linear regression of all of the data to 24 hours returns a pseudo-first order kinetic constant of 0.093 hr^{-1} ($r^2 = 0.976$), while regression over the first 7 hours gives a kinetic constant of 0.049 hr^{-1} ($r^2 = 0.946$) and regression over the last 17 hours gives a kinetic constant of 0.11 hr^{-1} ($r^2 = 0.993$). The r^2 values are reported only as an indication of the ability of the linear regression model to describe the data. They have no direct bearing on the appropriateness of the physical model of the system, in this case a first order reaction. Reference to the plotted data in Figure 52 shows that even though the r^2 value for the regression over the full set of data is quite high, the model fails to account for the shape of the curve.

The predicted progress curves using these constants in a first-order reaction model are shown in Figure 53, along with the PCP concentration data from Figure 50. Reliance on the early data tends to underestimate the progress of PCP transformation. Regression of the latter stages of the reaction overestimate the extent of reaction by a small amount. Regression of the full data set predicts the overall progress of the reaction quite well, except for the actual shape of the curve. In Figure 54 the kinetic constant derived from the full set of PCP data is used to model both the disappearance of PCP and the appearance of TeCP. The shape of the TeCP curve is not modeled well at all.



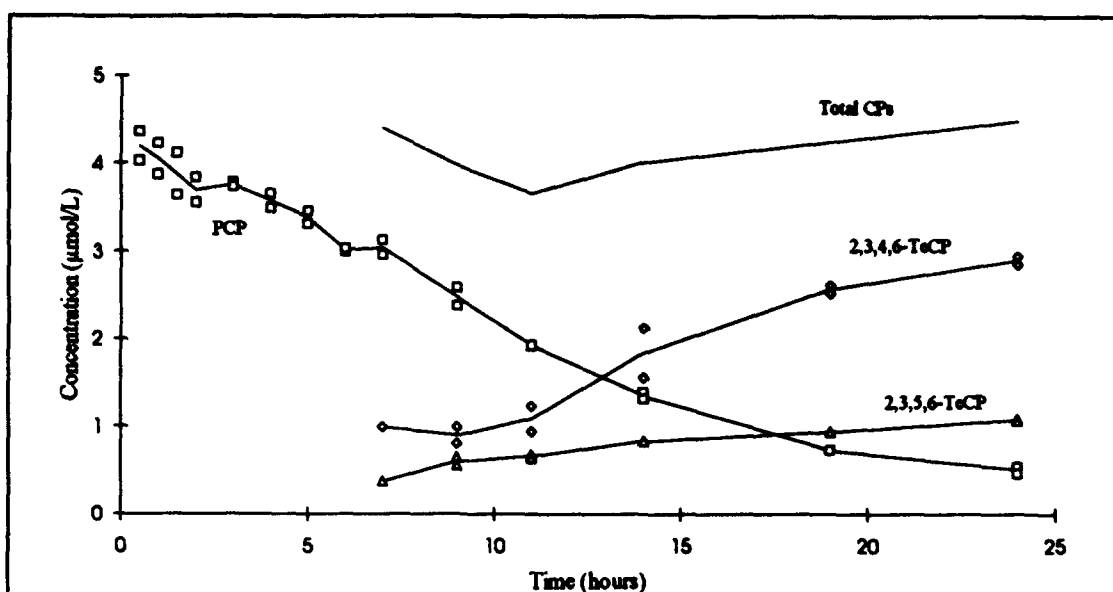


Figure 50. Progress curves for experiment 314. The experiment was conducted in a TCR with initial concentrations of 4.3×10^{-6} M PCP, 4.8×10^{-4} M vitamin B₁₂ and 0.005 M Ti(III) citrate. Analysis results from duplicate samples are shown. Lines connect the average values. Small amounts of TeCPs prior to seven hours were not quantifiable.

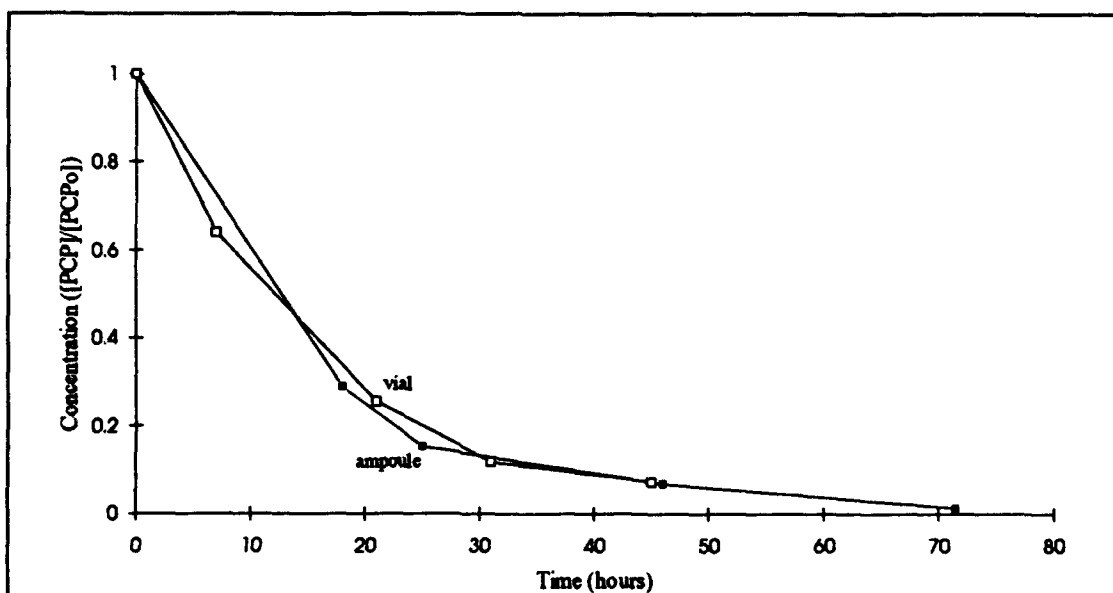


Figure 51. Progress curves for experiments 330c (ampoule) and d (serum vial). Due to slight differences in initial concentration, values are normalized to the initial PCP concentration to emphasize the similarity in rates.

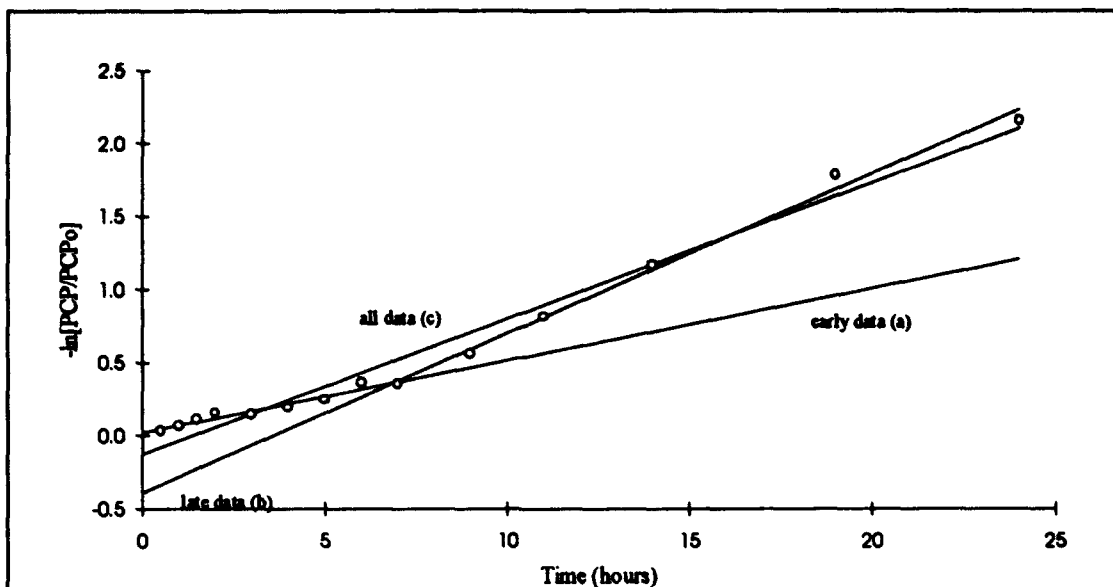


Figure 52. Data from Figure 50 transformed as a first order reaction. Nonlinearity of the resulting graph suggests that the first order model is invalid. The three lines are the result of linear regression of all the data (line c), or just the early (line a) or later (line b) portions.

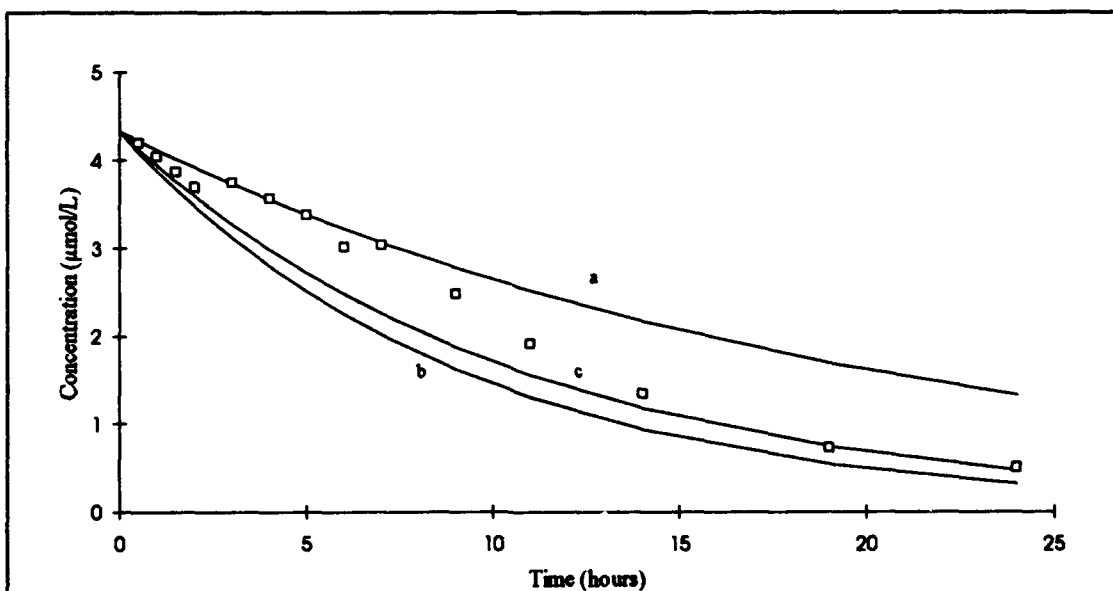


Figure 53. Predicted progress curve for PCP depletion in experiment 314. Linear regression of the first seven hours (a), the last 17 hours (b) or all of the data (c) from Figure 52 returned kinetic constants which were used to predict the course of the reaction.

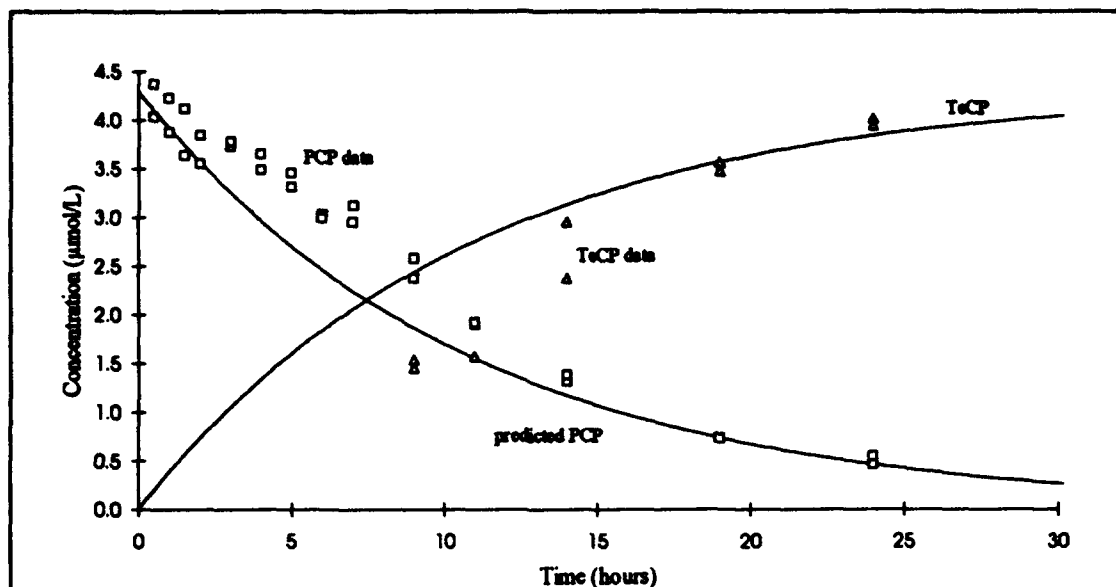


Figure 54. Data from experiment 314 modeled as a first order reaction. The value of k_1 was 0.093 hr^{-1} for the model. The extent of PCP decay is modeled well, but the shape of the PCP and TeCP curves is not.

The pseudo-first order rate constants returned from these seven experiments vary by a factor of three. The transformed data are shown in Figure 55. Table 9 summarizes all of the experiments and the first order rate constants returned from their evaluation. All of the data from experiments 330a through 330d were analyzed together by multiple regression analysis, hence the identical r^2 values (Appendix D). By considering the reaction conditions in Table 9, no clear trend is evident. The experiments with the two virtually identical highest rates (Experiments 249 and 306) had a two-fold difference in vitamin B_{12} concentration. Experiment 306 was conducted in February, when the laboratory temperature was only 18°C , while Experiment 249 was conducted in a temperature-controlled room at 25°C . Experiment 306 was exposed to ambient laboratory light, while Experiment 249 was foil-wrapped. The experiments with intermediate values again varied by twofold in vitamin B_{12} concentration, and in PCP concentration as well. One was foil-wrapped, and one was intentionally exposed to a lamp. The two slowest experiments (330c and 330d) were

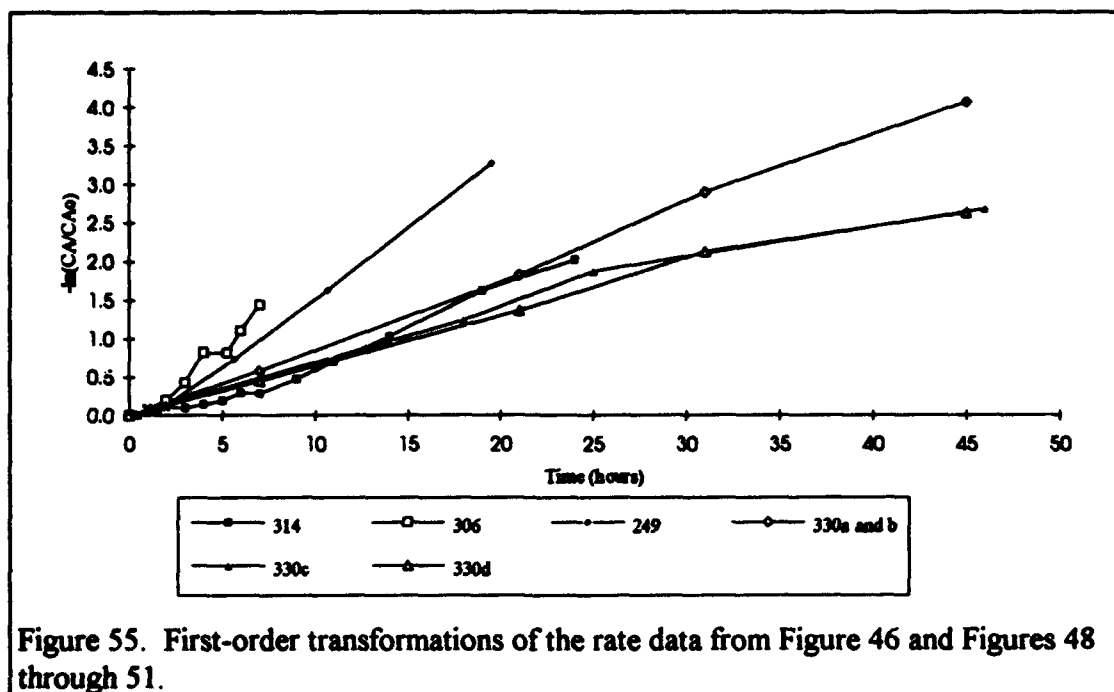


Table 9. Pseudo-first order rate constants and reaction conditions for several PCP reductive dechlorination experiments.

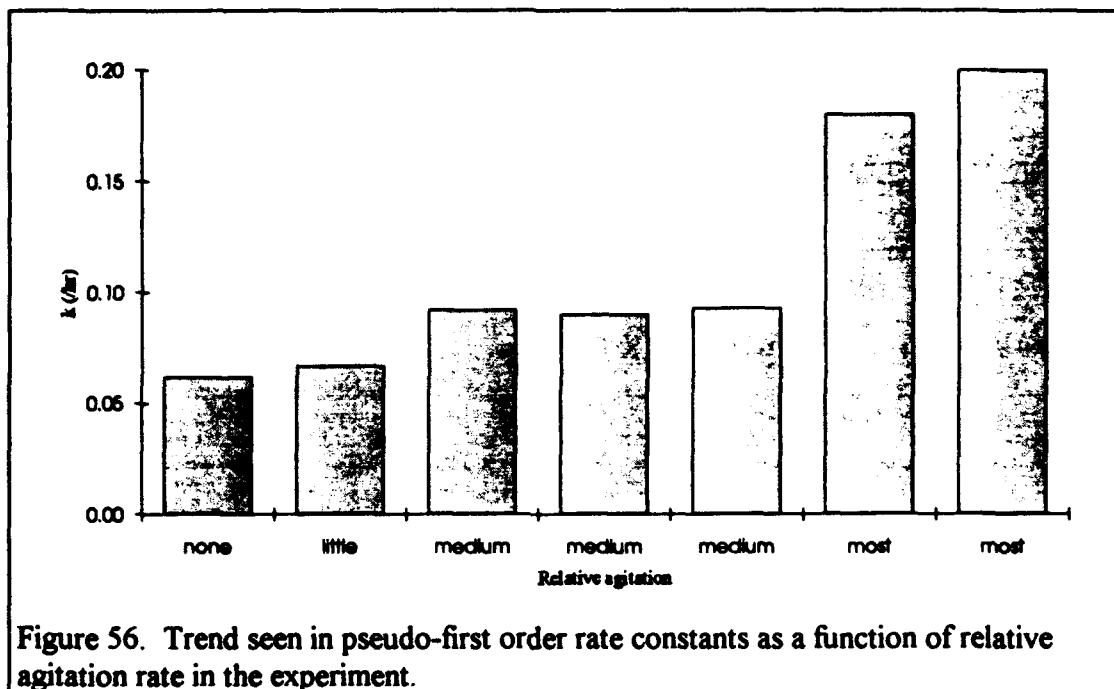
Exp #	B ₁₂ (mM)	PCP (μM)	Ti(III) citrate (M)	Light	Ambient Temp (°C)	Reactor, agitation ¹	Pseudo-first order rate constant k (hr ⁻¹)	r ²
249	1.0	2.2	0.20	dark	25	vial, 2	0.18	0.999
306	0.57	3.1	0.008	room	18	TCR, 2	0.20	0.959
314	0.48	4.3	0.005	room	22	TCR, 1	0.092	0.976
330a	0.91	9.0	0.024	dark	23	TCR, 1	0.090	0.994
330b	0.91	9.0	0.024	lamp	23	TCR, 1	0.093	0.994
330c	0.91	9.0	0.024	dark	27	ampoule, 0	0.062	0.994
330d	0.91	9.0	0.024	dark	23	vial, 0	0.067	0.994

¹Agitation level estimated from reaction records. No or little agitation, 0; maximum agitation 2; intermediate agitation 1

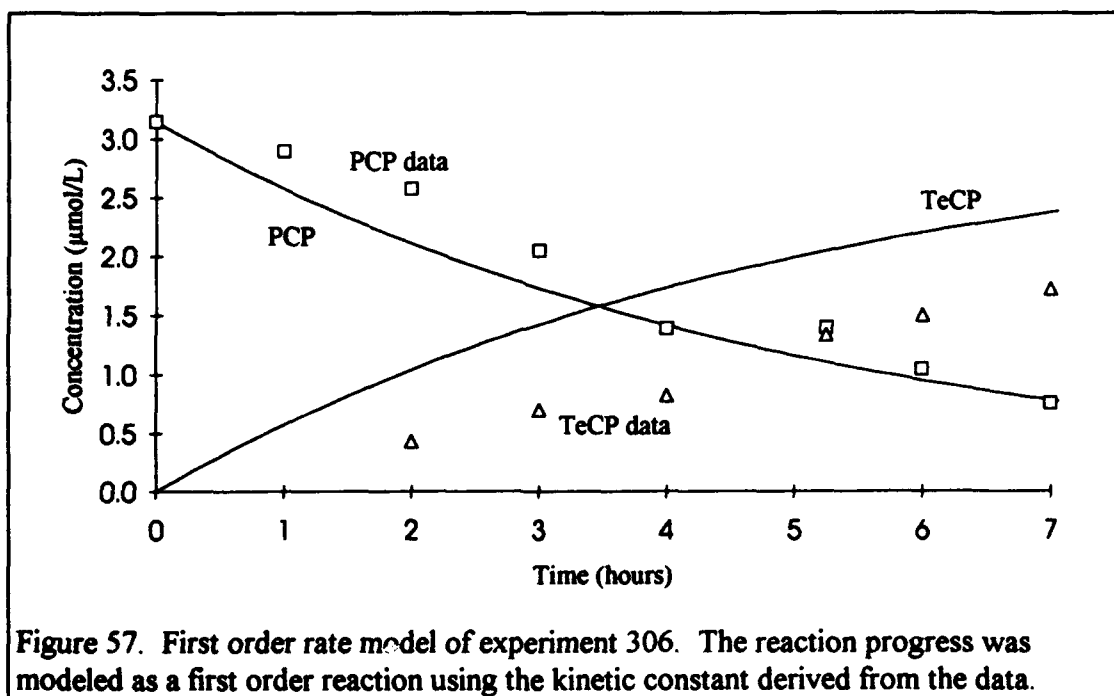
virtually identical in reactants to two of the intermediate rate experiments (330a and 330b). Neither temperature, light, nor concentration show a clear relationship to the observed rates.

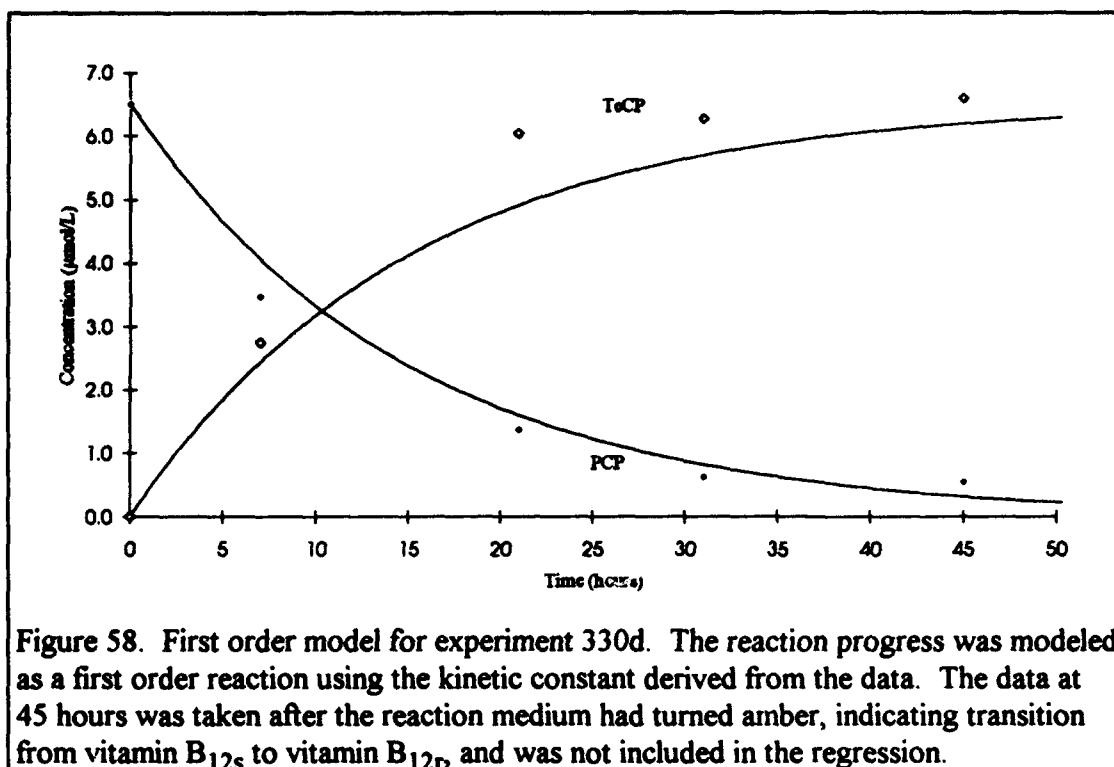
The recorded temperature for each reaction (Table 9) was the temperature of the room. The actual temperature of the reaction medium may have been somewhat higher. A thermometer placed in a culture tube with 3.0 mL water and stirring over the same stir plate as was used in these experiments registered 5°C higher than the ambient temperature of the room. Radiant heat from the lamp used in experiments 330a and b probably heated these even further. Experiment 249 was in the well of an empty shaker bath, and may have been subject to machine heat from the shaker. The trend does not continue for experiment 306, which should have been considerably cooler than the others, even if heated by the stir plate.

Another factor which seems to show a trend is agitation. These reactions are much too slow to be diffusion controlled (Hammes, 1978), and diffusion-controlled reactions are unaffected by mixing in batch reactors (Levenspiel, 1972). However, the two experiments which exhibited the highest rates also had the highest agitation rates. Experiment 249 was conducted in a serum vial mixed on a laboratory shaker, and experiment 306 was in a TCR centered over the center of a magnetic stirrer. Experiments 314 and 330a and b were conducted in TCRs positioned over the stir plate to accommodate other experiments. Experiment 330c was conducted in stationary ampoules, with no agitation. Experiment 330d was conducted in a serum vial with agitation provided by a flea-sized stir bar. The large diameter of the serum vial compared to the TCR, and the convex surface of the bottom of the serum vial probably resulted in much less mixing in this experiment than in any other except 330c. Figure 56 compares the rates to the degree of agitation.



Figures 57 and 58 model the reaction progress of experiments 306 and 330d, respectively, as first order reactions, using the rate constants from Table 9. The model overestimates the production of TeCP for experiment 306. The model underestimates the extent of PCP disappearance for experiment 330d.





Seven reductive dechlorination kinetic experiments were conducted which gave three distinct sets of kinetic constants. The fastest reactions were modeled with a kinetic constant of about 0.2 hr^{-1} , the slowest with a constant of about 0.06 hr^{-1} , and intermediate reactions with a constant of about 0.09 hr^{-1} . Reaction conditions were evaluated, and variations in room temperature, light, nor reactant concentrations could explain the variability. Some correlation with agitation, and a possible relationship to reaction temperature was detected. As was mentioned before, some of the progress curves followed a pattern of starting slowly, then proceeding more rapidly. Both reactions 249 and 306 exhibited this behavior, so that a more detailed study (experiment 314) was undertaken specifically to investigate this phenomenon.

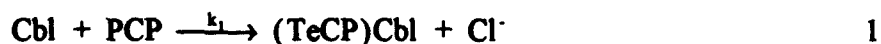
The general shape of these reaction progress curves often indicates an autocatalytic reaction. It was also recognized that this curve resembles the sum of the

initial reactant and first intermediate of a sequential first order reaction. Both models were investigated with experiment 314.

Autocatalytic model

Krone et al. (1989b) observed that, with Ti(III) citrate as reductant, methane was produced from carbon tetrachloride with methylcobalamin as catalyst at a rate faster than the rate of methane generation from methylcobalamin alone. They concluded that methylcobalamin could not be the only intermediate responsible for the ultimate production of methane. In the mechanism they proposed, chloromethane alkylates methylcobalamin to form a bis-methyl cobalamin as a second possible precursor to methane. In order for this mechanism to account for the accelerated production of methane from carbon tetrachloride, methylcobalamin would have to be a better catalyst than cobalamin. The formation of methylcobalamin from cobalamin would therefore fuel an autocatalytic process.

I have earlier proposed that the reductive dechlorination of chlorophenols by vitamin B₁₂s proceeds through an aryl cobalamin intermediate (Chapter 6). Bis aryl adducts of the cobalamin model compounds cobalt bis(salicylaldehyde) ethylenediimine (Costa et al., 1967) and cobalt bis(diacetylmonoxime-imino)propane 1-3 (Costa et al., 1969a, b) are known. The aryl cobalamin could stimulate an autocatalytic reaction if it were a better catalyst than free cobalamin. To test this hypothesis, a numerical model was constructed to simulate the reaction steps. The reaction was presumed to proceed as follows:



where Cbl is cobalamin (vitamin B₁₂), TeCP is tetrachlorophenol, and (TeCP)Cbl is the aryl cobalamin. In order to generate an autocatalytic species in the model, it is

necessary to include separate kinetic constants for the formation of aryl cobalamin (k_1) and the degradation of aryl cobalamin to TeCP and cobalamin (k_2). The reaction responsible for autocatalysis is reaction 3, which is assumed to be faster than reactions 1 and 2. The first reaction is assumed to be pseudo-first order in PCP, the second reaction is first order, and the third reaction is second order. It is also assumed that excess reductant is always available and that the rate of reduction of cobalamin species is insignificant to the course of the overall reaction.

A numerical model was constructed to simulate this reaction mechanism. Using an optimization function in a commercial spreadsheet program (Excel Solver, Microsoft Corporation, Bellevue, WA), the three constants were determined for a least sum of squares algorithm for the predicted PCP curve. The optimum solution was $k_1 = 0.033$, $k_2 = 0$, and $k_3 = 4.9 \times 10^4$. While this solution predicted the PCP curve fairly well (Figure 60), it grossly underestimated the production of total tetrachlorophenols. In addition, this model predicted that the arylcobalamin would not cleave to form TeCP ($k_2 = 0$, or, at least, $k_2 \ll k_3[\text{PCP}][(\text{TeCP})\text{Cbl}]$), so that the arylcobalamin was the only active catalytic species under the reaction conditions. Organocobalamins are frequently much less stable in the presence of oxygen than they are under anaerobic conditions (Schrauzer, 1968). If, on analytical workup of the sample, it is assumed that the arylcobalamin decomposes to TeCP and cobalamin, then the TeCP analytical value would equal the combined concentrations of TeCP and arylcobalamin. Figure 60 shows the result of this assumption on the predicted progress curves of PCP and total TeCPs. This model fits the data quite well.

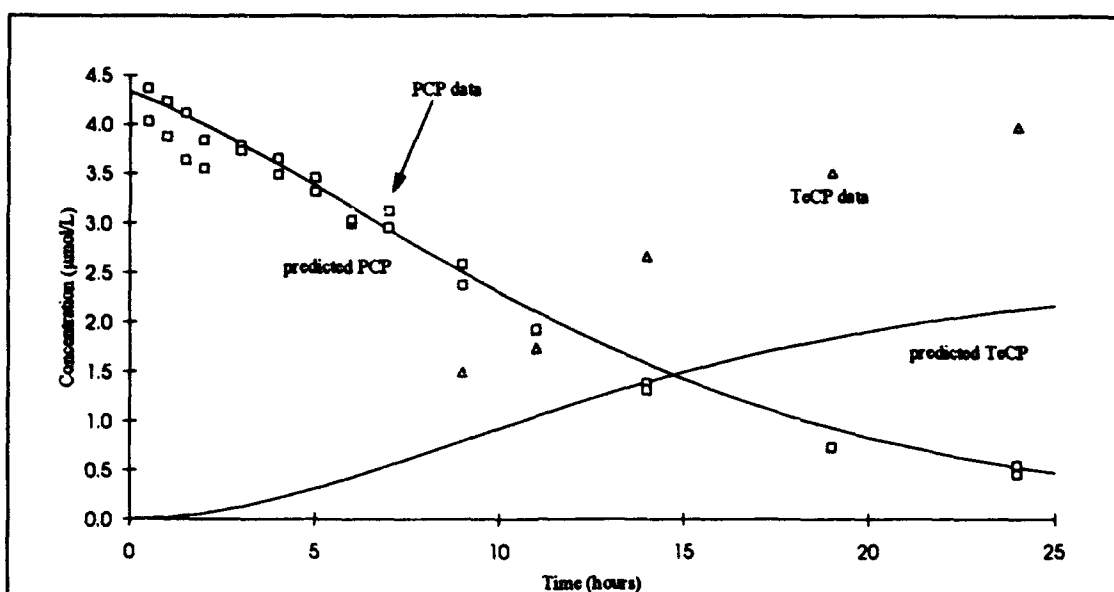


Figure 59. Autocatalytic model of vitamin B_{12s} reductive dechlorination of PCP. Using a numerical model of an autocatalytic process optimized to fit the PCP data, the PCP and TeCP rate profiles for experiment 314 were predicted. The model grossly underestimates the production of tetrachlorophenols.

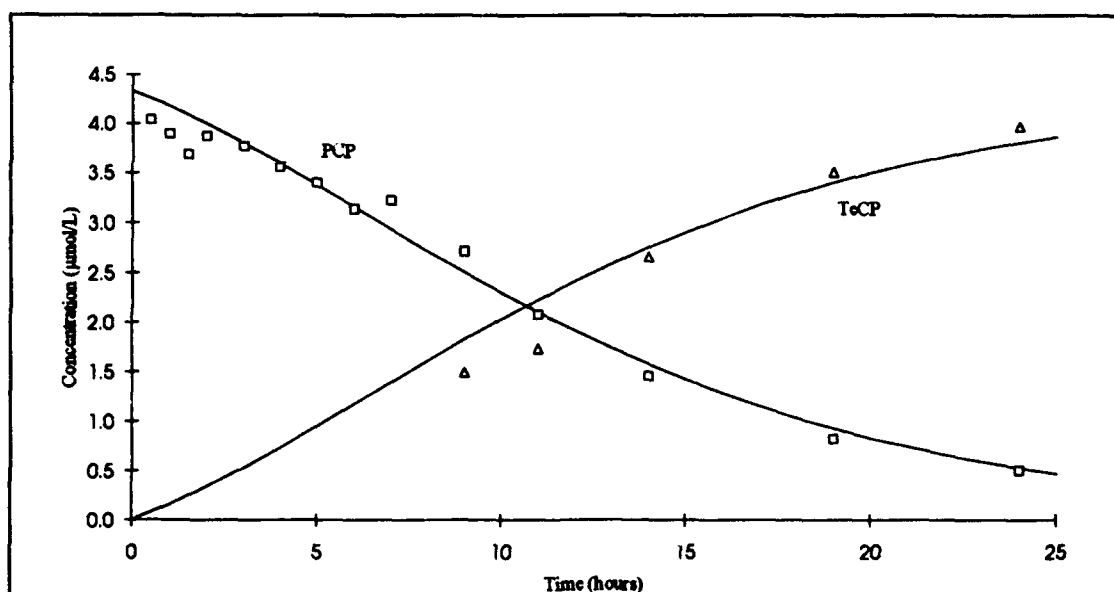
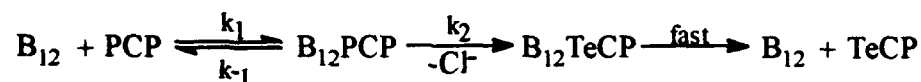


Figure 60. Modified autocatalytic model of vitamin B_{12s} reductive dechlorination of PCP. This model is similar to that shown in Figure 60, but assumes that the autocatalytic arylcobalamin species is cleaved to TeCP when the sample is processed.

Artifact model

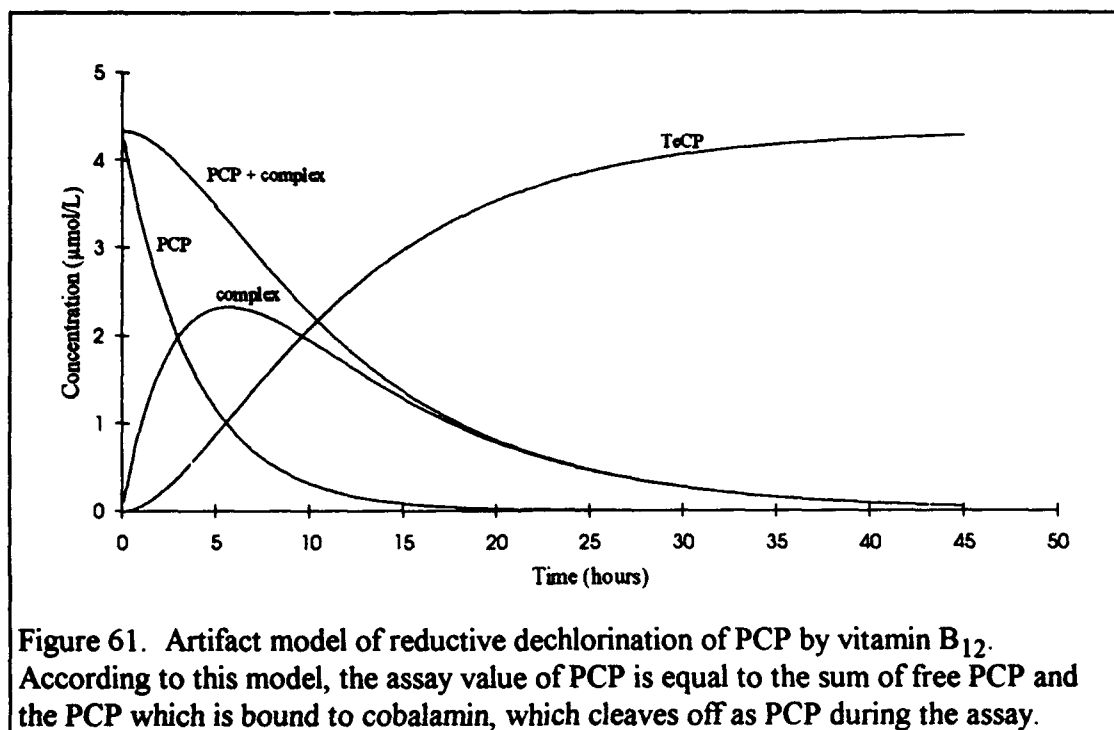
The third possibility considered was that the curvature represented an artifact of the assay procedure. I have earlier shown (Chapter 6) that the regiospecificity of vitamin B₁₂s reductive dechlorination is consistent with the formation of a metastable intermediate, a σ -complex between the arene and the cobalamin. Until the chlorine leaves, the complex could revert to vitamin B₁₂s and PCP. If the elimination of the chlorine were the rate-limiting step, and if the preparation of the sample for analysis cleaved the σ -complex into vitamin B₁₂ and PCP, then the value of the PCP assay would equal the sum of the concentrations of PCP and σ -complex. The overall mechanism would be



where B₁₂ is vitamin B₁₂, B₁₂PCP is the σ -complex, and B₁₂TeCP is the arylcobalamin. If $k_{-1} < k_2$, and the second reaction is the rate limiting step, B₁₂PCP would accumulate until the course of the reaction reflected only the chloride elimination step.

A numerical model was constructed to simulate this mechanism. Toward the end of the reaction, most of the initial reactant should be gone, and any PCP in the assay should be due only to the complex. Therefore, the rate constant k_2 was chosen as the rate of the latter part of Experiment 314. The value of k_1 was chosen with the aid of the Excel Solver program to optimize a least squares fit of the data to the sum of PCP and complex. For clarity, Figure 61 shows the predicted concentrations for experiment 314 without the data being plotted. Figure 62 includes the data from experiment 314. This model fits this data set at least as well as does the modified autocatalytic model (Figure 60). The assumption that k_2 reflects the latter portion of the data is supported by the confluence of the complex curve and the PCP + complex curve. The assay value is equal to the PCP + complex curve.

While the artifact model fits the individual data set from experiment 314 very well, it does not model the other data sets using the same parameters. There is the same difficulty fitting the model to the data as exists with the first order model. In the end, the first order model with multiple measured rates is just as predictive, if not as descriptive, as the artifact model.



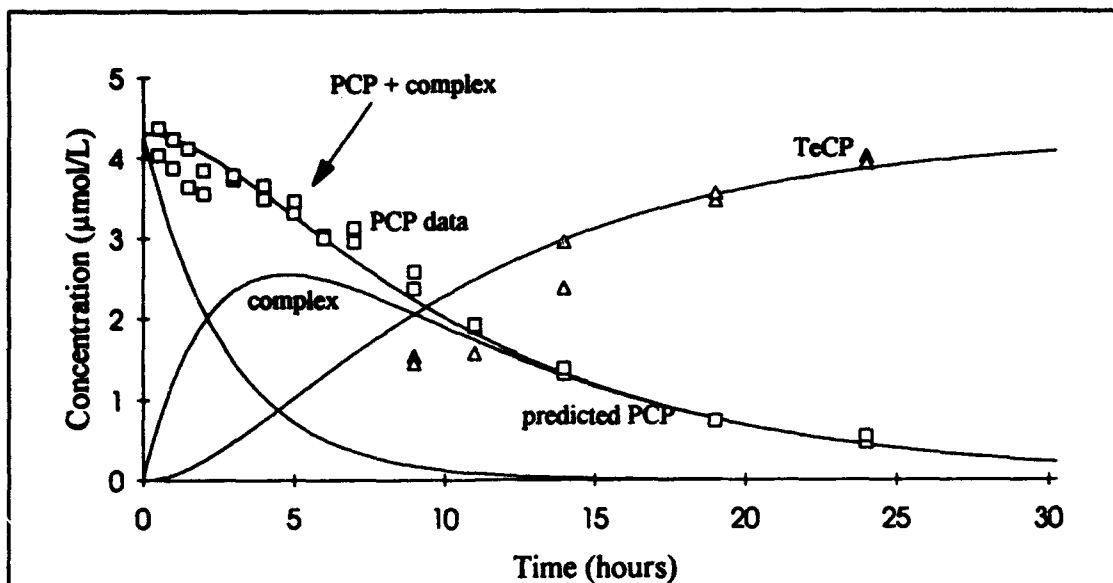


Figure 62. Artifact model with data from experiment 314 juxtaposed for comparison. The model fits this data set very well.

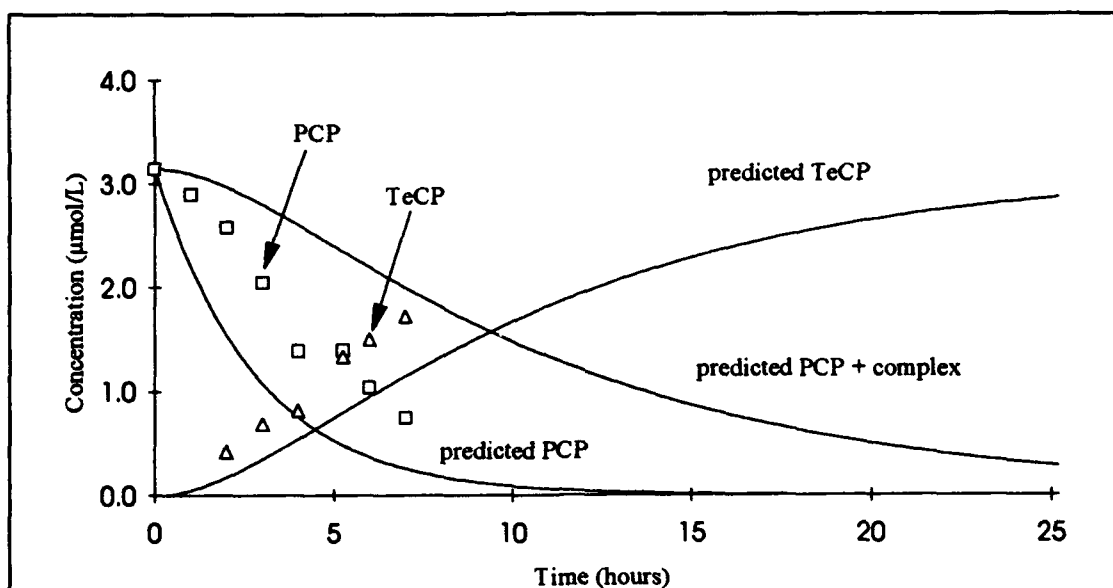


Figure 63. Experiment 306 with predicted progress curves using the artifact model and kinetic constants chosen to fit experiment 314.

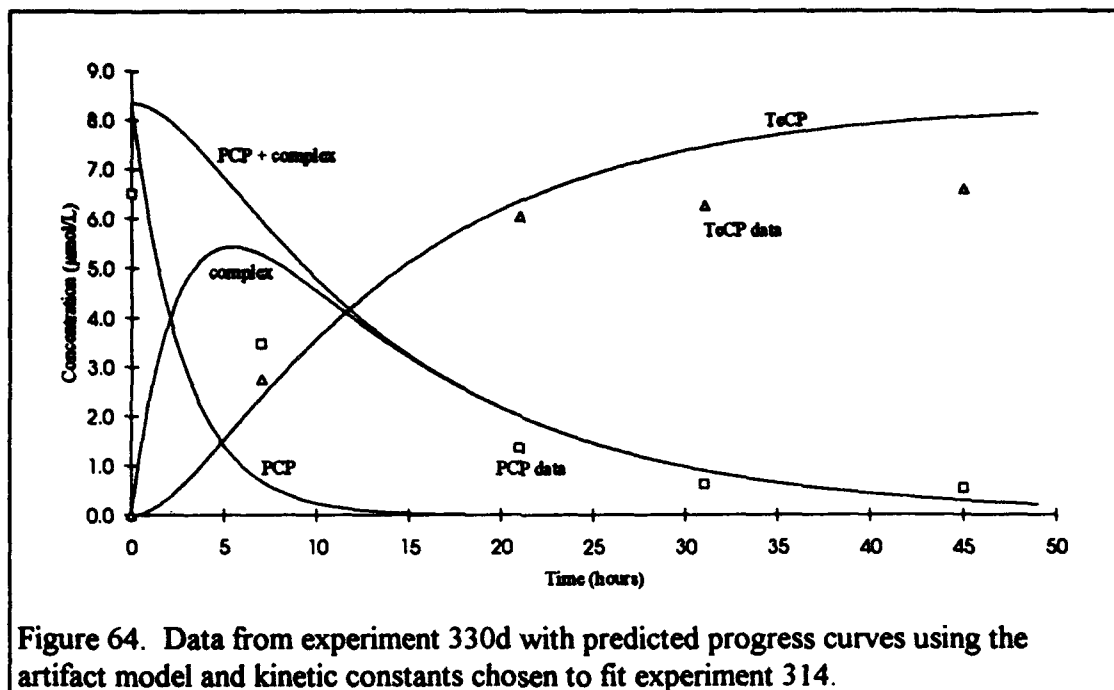


Figure 64. Data from experiment 330d with predicted progress curves using the artifact model and kinetic constants chosen to fit experiment 314.

The kinetics of this simple system have proven to be more complex than was originally assumed. Several experiments encompassing a variety of reagent concentrations and reaction conditions have resulted in a variety of results, all of which can not be explained with a single model. A simple first order model is predictive of the course of reaction, and describes well the experimental progress curves of some of the experiments. The measured first order kinetic constants from these experiments vary by nearly three fold. For some experiments the progress curves are not well described by a simple first order rate equation, but are better fit by a model which results from the addition-elimination mechanism of the chemical reaction. In this model, the analytical value of PCP results from both free PCP and the intermediate σ -complex, which decomposes during the analytical procedure to vitamin B₁₂ and free PCP. This model fits many of the data, but all experiments cannot be described with a single set of kinetic constants with this model or with a simple first order model. A model of an autocatalytic process was attempted, but this model failed to describe both PCP disappearance and TeCP appearance simultaneously.

Implications of vitamin B₁₂ kinetics to a biological system

The kinetics and products of reductive dechlorination by vitamin B₁₂s resemble those observed by Nicholson et al. (1992) for a pentachlorophenol-acclimated anaerobic microbial consortium in batch experiments. This consortium was able to reductively dechlorinate PCP, at similar concentrations as the present study, to primarily 2,3,5,6-TeCP in about one day. Vitamin B₁₂s, at the fastest rates observed in the current study, could duplicate this rate. Although an anecdotal effect on the reaction rate by agitation was observed, the temperature of reaction is the more likely source of rate variability. The rate at 35°C, the same used by Nicholson et al., should be even faster.

To compare the rates observed in the present study to those observed by Nicholson et al., an estimate of the vitamin B₁₂ content of their system must be made. Mazumder et al. (1987) have measured the corrinoid content of a batch culture of *Methanosarcina barkeri* Fusaro at 1.35 mg/L with a dry cell density of 0.66 g/L. That is approximately 1×10^{-6} M vitamin B₁₂ assuming that all of the corrinoid expresses identical activity. Approximately 40% of the corrinoid was released free to the culture fluid.

In the Nicholson et al. reactor, vitamin B₁₂ was initially present at 6.6×10^{-10} M, but CoCl₂·6H₂O was included in the culture medium at 16.7 mg/L, sufficient to provide at most up to 7×10^{-5} M vitamin B₁₂. Mazumder et al. reported, however, that concentrations of Co salts above about 0.6 mg/L CoCl₂·6H₂O could only be attained if Na₂S in the medium were replaced with cysteine to prevent precipitation of the heavy metal sulfide. They achieved maximum corrinoid production with 9.6 mg/L CoCl₂·6H₂O and cysteine broth in batch culture at 2×10^{-6} M. This level is still two to three orders of magnitude less than the vitamin B₁₂ concentrations used in the present study. The culture broth of Nicholson et al. contained Na₂S, so it is doubtful whether

all of the Co added would be available to the organisms. Mazumder et al. found that $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ concentrations above 9.6 mg/L inhibited microbial growth. The vitamin B_{12} level in the Nicholson et al. reactor was probably in the region of $1 \times 10^{-6} \text{ M}$. This level of vitamin B_{12} is two to three orders of magnitude less than the levels used in the present study. They are also of the same magnitude as the challenging concentrations of PCP. Under these circumstances, the pseudo-first order approximation would no longer hold, and the observed rate of reductive dechlorination would be critically dependent upon corrinoid concentration, and would be considerably slower than the rates observed here. Therefore, the simple presence of vitamin B_{12} reduced to vitamin $\text{B}_{12\text{s}}$ in the cell could not account for the rates of reductive dechlorination observed by Nicholson et al.

It is interesting to note, however, that the general shape of the PCP decay curve observed by Nicholson et al. resembles that seen in experiments 249, 306, and 314 in the present study (Figures 48, 49, and 50). Figure 65 shows the PCP decay data for a batch experiment with an anaerobic microbial consortium that had been acclimated to PCP for ten days (Nicholson et al., 1992), and predicted decay curves for first order reactions with kinetic constants of 0.09 and 0.2 hr^{-1} for comparison. The data follow the same general trend of a period of slow decay, resembling a lag phase, followed by more rapid loss of PCP.

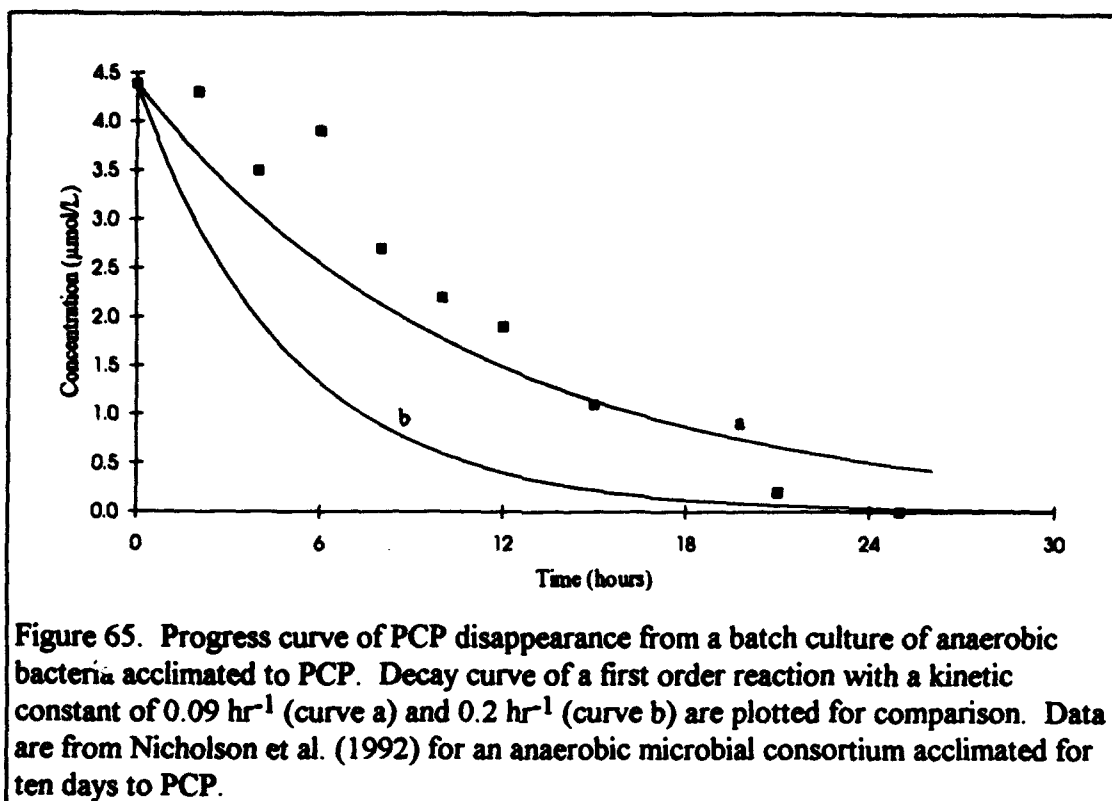


Figure 65. Progress curve of PCP disappearance from a batch culture of anaerobic bacteria acclimated to PCP. Decay curve of a first order reaction with a kinetic constant of 0.09 hr^{-1} (curve a) and 0.2 hr^{-1} (curve b) are plotted for comparison. Data are from Nicholson et al. (1992) for an anaerobic microbial consortium acclimated for ten days to PCP.

Conclusions

Vitamin B_{12s} reductively dechlorinates PCP by a mechanism which is insensitive to light. The progress of the reaction can be predicted by pseudo-first order kinetics when vitamin B₁₂ is present in great excess, with a kinetic constant of 0.06 to 0.2 hr^{-1} . The variability in the rates was anecdotally dependent upon agitation, but is most likely dependent upon the temperature of the reaction mixture. While a first order kinetic model is predictive of the extent of reaction, it does not always describe the reaction progress curves. Under certain conditions, the progress curves are best described as an artifact of the assay procedure, and reflects the molecular mechanism of the reaction.

Based upon the reductive dechlorination rates observed in this study, the simple presence of vitamin B₁₂ in a reaction medium cannot account for observed rates of biological reductive dechlorination.

Chapter 8

Effect of Reducing Conditions on the Reductive Dechlorination of Pentachlorophenol by Vitamin B₁₂

Abstract

Methods were developed to study the ability of vitamin B_{12r} to reductively dechlorinate pentachlorophenol. Vitamin B₁₂ was reduced to vitamin B_{12r} with dithiothreitol or limiting amounts of Ti(III) citrate and sealed in glass ampoules for four months. Under the conditions of this study, no reductive dechlorination of pentachlorophenol was observed.

Introduction

Pentachlorophenol (PCP) is degraded by anaerobic microbial consortia by a process of reductive dechlorination, whereby a chlorine is removed from the aromatic ring as chloride and replaced with a hydrogen atom, while the carbon to which the chlorine was attached is concomitantly reduced by two electrons. Anaerobic microbial consortia unacclimated to PCP remove preferentially the chlorine *ortho* to the hydroxyl, while PCP-acclimated consortia also remove the *para* and *meta* chlorines. The *ortho* chlorines of the lesser-chlorinated chlorophenols are relatively more susceptible to microbial attack, even by consortia acclimated to PCP (Nicholson, 1992).

Several naturally-occurring metalloorganic compounds have been shown to catalyze reductive dechlorination, and the implications to natural processes have been recognized. Carbon tetrachloride, perchloroethylene and their dechlorinated congeners are reductively dechlorinated by the cobalt corrinoid vitamin B₁₂, the nickel porphinoid

coenzyme F₄₃₀, and the iron porphyrin heme, with titanium(III) citrate or dithiothreitol as electron donor (Krone et al., 1989a, b; Gantzer and Wackett, 1991). Vitamin B₁₂ can also catalyze the reductive dechlorination of the pesticides lindane (Bienie et al., 1970; Marks et al., 1989), mirex and kepone (Schrauzer and Katz, 1978), and the aromatic halides hexachlorobenzene and pentachlorobenzene (Gantzer and Wackett, 1991; Assaf-Anid et al., 1992), 2,3,4,5,6-pentachlorobiphenyl (Assaf-Anid et al., 1992), and several chlorophenols (Gantzer and Wackett, 1991; Chapter 6).

In contrast to the regiospecificity of reductive dechlorination expressed by anaerobic bacteria, vitamin B_{12s}, with Ti(III) citrate as electron donor, reductively dechlorinates PCP preferentially at the *meta* and *para* positions (Gantzer and Wackett, 1991; Chapter 6). An addition-elimination mechanism has been proposed (Chapter 6), whereby vitamin B₁₂, reduced to the supernucleophile vitamin B_{12s} (cob(I)alamin) by Ti(III) citrate, attacks the ring carbon and displaces the chlorine by nucleophilic aromatic substitution, simultaneously reducing the carbon. Subsequent cleavage of the Co-C bond and abstraction of a hydrogen results in the reductively dechlorinated product.

Reductive dechlorination of 2,3,4,5,6-pentachlorobiphenyl (Assaf-Anid et al., 1992) using dithiothreitol as the reducing agent, led us to suspect that an alternative mechanism may also be expressed by vitamin B₁₂. When reduced with dithiothreitol (DTT), vitamin B₁₂ solutions are yellow (Assaf-Anid et al., 1992), which indicates incomplete reduction to vitamin B_{12r} (cob(II)alamin) (Schneider and Stroinski, 1987; Pratt, 1972).

DTT supports reductive dechlorination of alkyl halides by vitamin B₁₂ at rates slower than the rates supported by stronger reductants, such as Ti(III) citrate (Krone et al., 1989b; Gantzer and Wackett, 1991). Vitamin B_{12r} reacts with alkyl halides by a radical mechanism to form the alkyl cobalamin and halide anion (Halpern and Maher,

1965; Blaser and Halpern, 1980). The thesis of this investigation was that a similar mechanism could exist with aryl halides, and that such a mechanism would exhibit different regiospecificity. In this study, the reductive state of the vitamin B₁₂ was examined to determine its possible effects on the regiospecificity and kinetics of the reductive dechlorination of PCP.

Materials and Methods:

All reactions were conducted in 0.66 M Tris (Life Technologies, Inc., Gaithersburg, MD) buffer, pH 8.2, except as otherwise noted. Sources of reagents were as follows: Vitamin B₁₂ and PCP (99.9% purity) were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,4,6- and 2,3,5,6-tetrachlorophenols (TeCP) were purchased from Ultra Scientific, Inc. (N. Kingston, RI.), 95+% purity. A stock solution of 250 mM Ti(III) citrate in 0.66 M Tris buffer, pH 8.2, was prepared from Na₃ citrate (Mallincrodt, Inc., St. Louis, MO) and TiCl₃ solution (Fluka Chemical Corp., Ronkonkoma, NY) as previously described (Chapter 5). Stock solutions of the CPs were made in methanol and diluted in Tris buffer for transfer to the reaction mixtures. Analysis of reaction products was by gas chromatography as previously described (Chapter 5).

Reduction of vitamin B₁₂ by DTT and limited amounts of Ti(III) citrate

One mL of a 5×10^{-3} M vitamin B₁₂ solution in a 2-mL glass ampoule (Wheaton Scientific, Millville, NJ) was purged with purified argon for ten minutes, after which a quantity of Ti(III) citrate stock solution was added by microsyringe and the ampoule sealed in a gas flame. The absorption spectra of the resulting solutions were recorded with a Hewlett-Packard model 8452A recording spectrophotometer.

Attempted reduction of PCP by vitamin B₁₂

All reactions were conducted in hermetically-sealed, 2-mL glass ampoules. Because of a report of reductive dechlorination of the aromatic compound 2,3,4,5,6-pentachlorobiphenyl (Assaf-Anid et al., 1992) by vitamin B₁₂ using DTT in a solvent of 50% dioxane and 50% Tris buffer, similar conditions were chosen for this experiment. In order to increase the likelihood of observing reductive dechlorination products, a high concentration of PCP was used. A solution of 3×10^{-3} M PCP and 5×10^{-4} M vitamin B₁₂ and was prepared in 50% dioxane and 50% 0.66 M Tris buffer, pH 8.2. To a portion of this solution, sufficient DTT was added to a final concentration of 125 mM. This solution was purged with argon and dispensed in 1 mL aliquots to a series of ampoules. Each ampoule was further purged for ten minutes, then flame sealed.

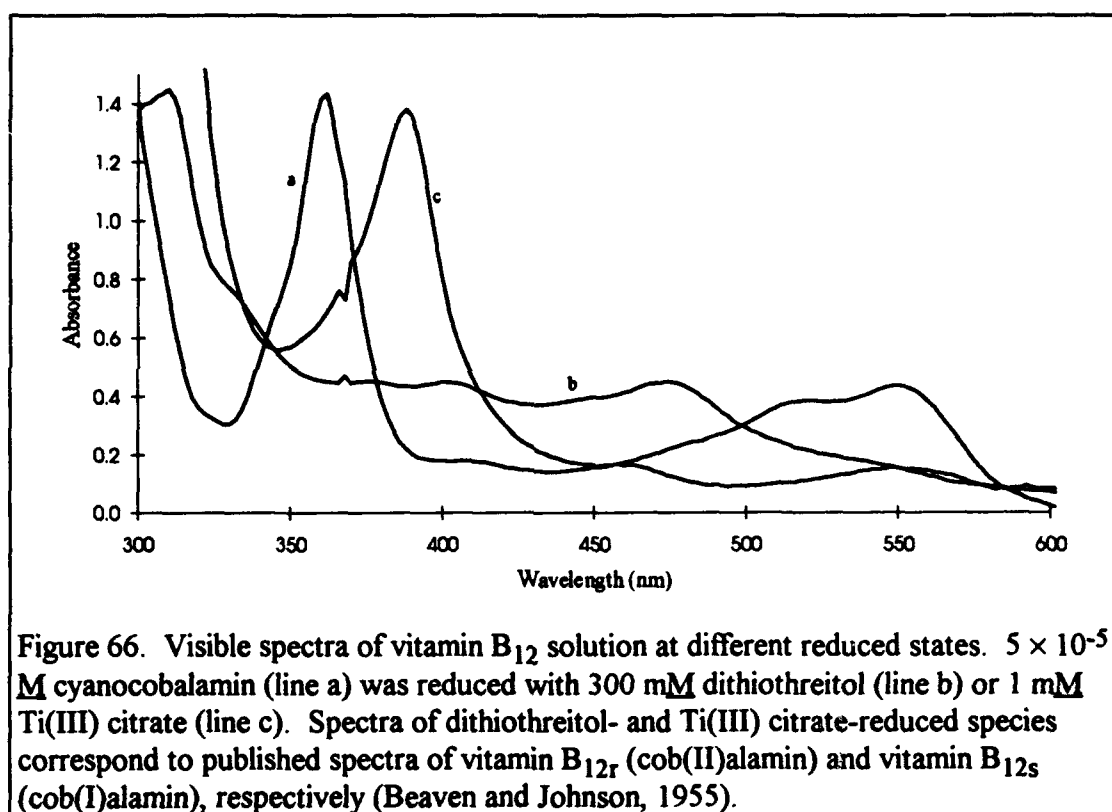
A separate portion of the PCP-vitamin B₁₂ solution was dispensed in one mL aliquots to ampoules. Each ampoule was individually purged with argon for ten minutes, and three μ L of stock Ti(III) citrate was added. Both the DTT and Ti(III) citrate-reduced solutions were amber in color. Spectra were not obtained due to the color intensity of the solutions. The ampoules were incubated in the dark at 27°C. Ampoules were chosen at random for assay throughout the experiment.

In order to determine whether unionized PCP might be more susceptible to reductive dechlorination by vitamin B₁₂, a similar set of experiments was prepared, but in 0.5 M acetate buffer, pH 5.0. Initial concentrations of reactants were 1×10^{-3} M vitamin B₁₂, 1.2×10^{-4} M DTT, and 1.0×10^{-5} M PCP. Control ampoules were prepared with PCP and DTT only at the same concentrations.

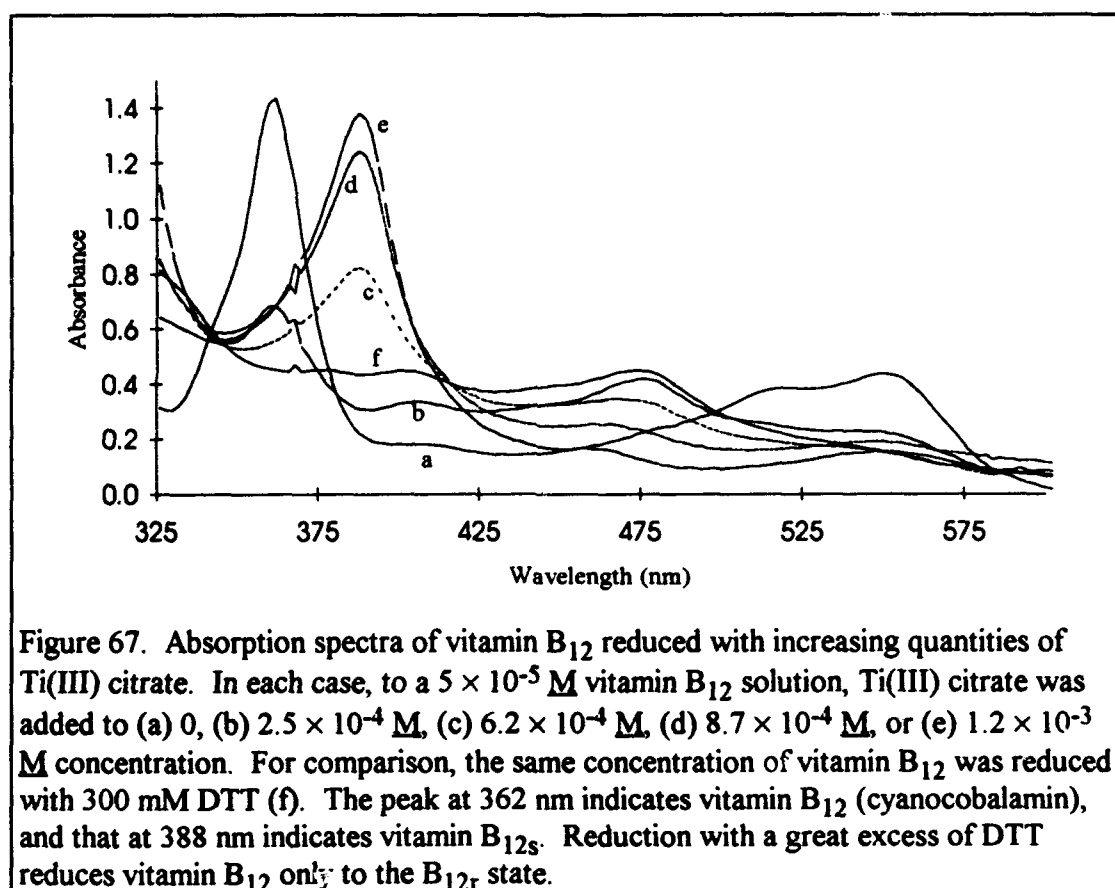
Results and Discussion

Figure 66 shows the visible spectra of 5×10^{-5} M vitamin B₁₂ solutions with no reductant and reduced with excess DTT or Ti(III) citrate in 0.66 M Tris buffer, pH 8.2. Comparison with published spectra shows that even with 300 mM DTT, this reductant

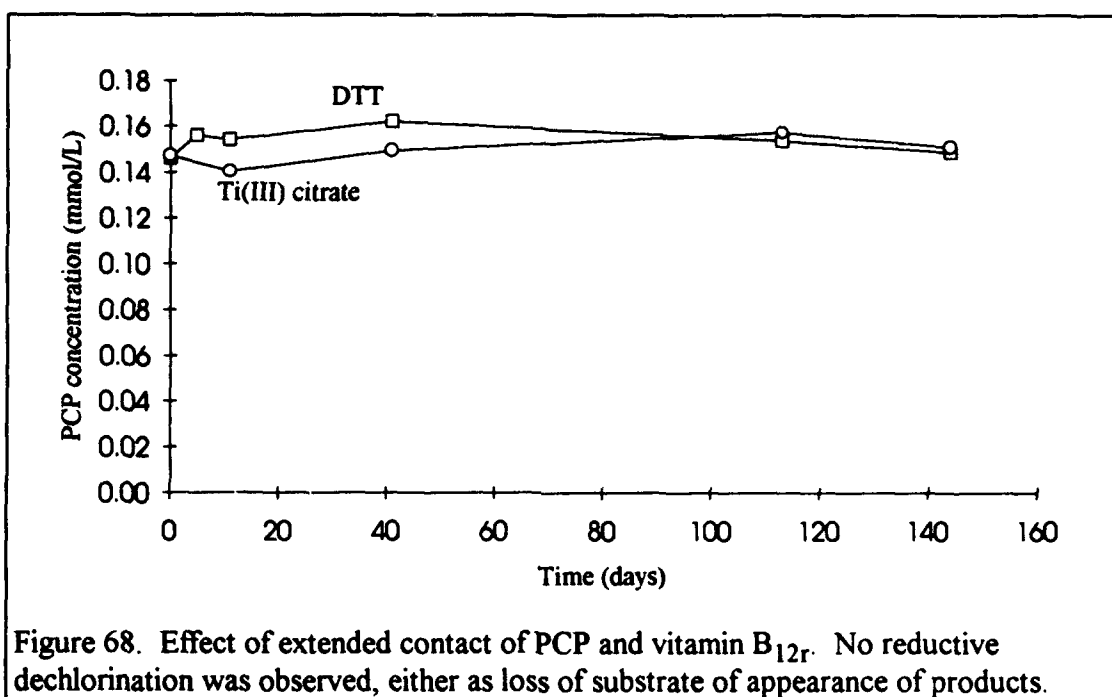
can only reduce vitamin B₁₂ to the Co(II) oxidation state, or vitamin B_{12r}. Ti(III) citrate effectively reduces vitamin B₁₂ to the Co(I) oxidation state, vitamin B_{12s}. While vitamin B_{12r} can potentially disproportionate into vitamin B_{12a} and vitamin B_{12s}, the equilibrium greatly favors vitamin B_{12r} (Pratt, 1972, p. 192). Any reductive dechlorination supported by DTT can, therefore, be attributed strictly to the reactivity of vitamin B_{12r}.



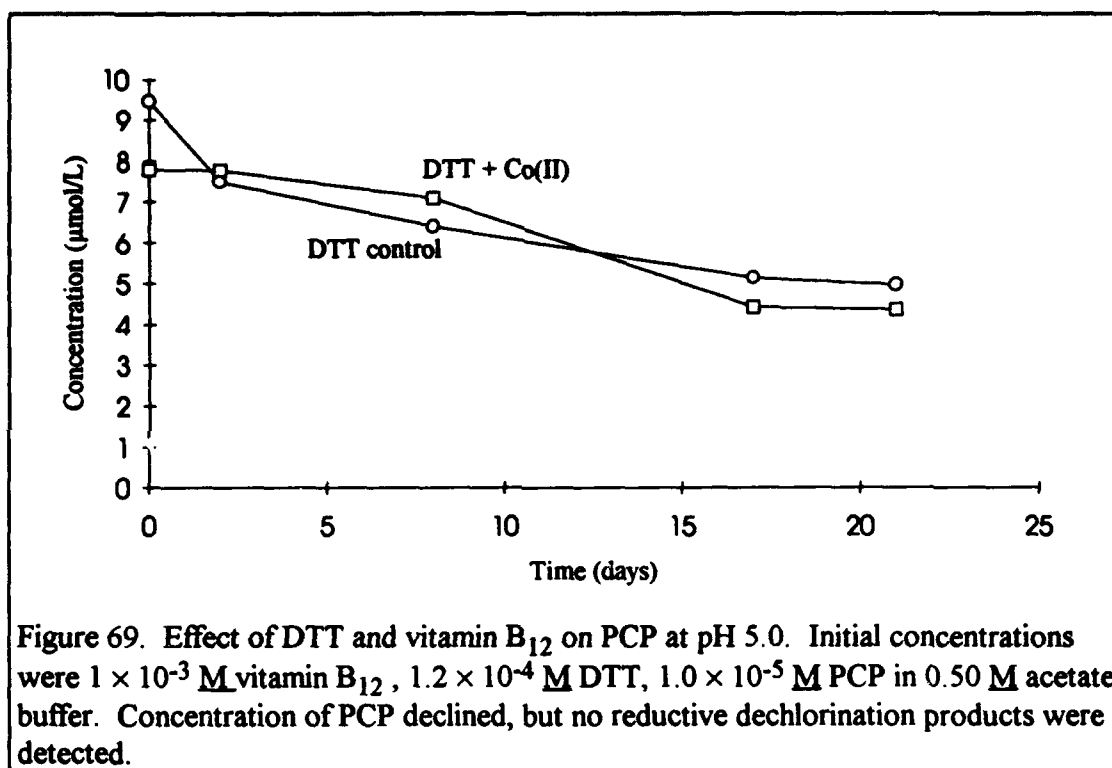
To isolate the activity, if any, of vitamin B_{12r} strictly to the cobalamin, vitamin B_{12} solutions were reduced with limited amounts of Ti(III) citrate. Figure 67 shows the absorbance spectra of vitamin B_{12} solutions reduced with varying concentrations of Ti(III) citrate, and with 300 mM DTT. Vitamin B_{12} is progressively reduced to mixtures of Co(III) and Co(II) species or Co(II) and Co(I) species by increasing concentrations of Ti(III) citrate. A small excess of Ti(III) citrate will, therefore, generate vitamin B_{12s} .



Even after 111 days of incubation, PCP was not reductively dechlorinated by vitamin B_{12r}, whether generated by DTT or by limited concentration of Ti(III) citrate (Figure 68). All solutions remained amber. The chlorine atoms are evidently held too strongly by the aryl group to participate in the reaction described above. Under the conditions of this study, reductive dechlorination of PCP is not supported by DTT.



In acetate buffer at pH 5.0, PCP concentration declines in the presence of 125 mM DTT with or without vitamin B₁₂ (Figure 69). No reductive dechlorination products were detected by gas chromatography. The fate of PCP under these circumstances is unknown.



Summary

Vitamin B₁₂, reduced to vitamin B_{12r} by DTT or limited amounts of Ti(III) citrate, did not reductively dechlorinate PCP at pH 8.2. PCP was degraded to unknown products by DTT at pH 5.0, with or without vitamin B₁₂.

If vitamin B₁₂ is involved in reductive dechlorination of PCP by anaerobic bacteria or microbial consortia, then it must be fully reduced to the vitamin B_{12s} state. Vitamin B₁₂ can be fully reduced to this oxidation level by FAD or NADH (Huheey, 1972). The ability of natural or engineered systems to reductively dechlorinate aromatic compounds may be encouraged by ever lower reducing potentials, which foster the reduced species necessary for the biochemical steps involved.

Chapter 9

Summary

The cobalamins are a fascinating family of molecules whose chemistry has been intensively studied for 45 years, yet continue to provide surprises. The ability of cobalamins reversibly to form carbon-cobalt bonds and thereby participate in a variety of organic reactions make them not only useful but essential to organisms for a myriad of biochemical processes.

Vitamin B₁₂ and related cobalamins have long been known to be involved in biological oxidation and reduction reactions, playing important roles in single-carbon chemistry, as well as other biological functions. It is becoming increasingly apparent that the unique chemistry of these organometallic molecules also permit the bioprocessing of anthropogenic pollutants in nature and in engineered systems.

The purpose of this research was to extend the understanding of the possible role of vitamin B₁₂ in the biological process of reductive dechlorination, specifically of aromatic halides. Toward that goal, the following objectives were established:

1. Determine the dechlorination pathways of the model catalytic system, using Ti(III) citrate as the source of electrons.
2. Obtain kinetic data from the model catalytic system and use the data to model the progress of the reductive dechlorination of pentachlorophenol.
3. Compare the kinetics and pathways of vitamin B₁₂-catalyzed reductive dechlorination to those for biological processes.
4. Determine the effect of reducing potential, i.e., the valence state of the vitamin B₁₂, on kinetics and pathways.

In pursuit of these objectives, certain procedural limitations had to be overcome, new techniques were developed, and existing techniques were improved. The chlorophenol assay procedure was recognized as cumbersome and laborious, so a miniaturized method was developed (Appendix A) which conserved resources, saved time, and improved efficiency. With the improved assay procedure in place, a vast number of individual reductive dechlorination experiments could be performed.

It was early on discovered that reductive dechlorination could not be supported by the first reductant tried, dithiothreitol (DTT). The reaction of DTT with vitamin B₁₂ produced an amber solution, which corresponded to literature descriptions of the single-electron reduction product, vitamin B_{12r}. When a stronger reductant, Ti(III) citrate, was used, the vitamin B₁₂ solution turned blue, corresponding to literature descriptions of the fully-reduced vitamin B_{12s}, and reductive dechlorination of PCP was observed. The course of the reaction was short-lived however, because the solution quickly turned from blue to amber, and the reaction ceased. It was recognized that the reactor system used (serum vials with Teflon/rubber septa) had serious limitations. Extensive trial and error experimentation, as well as systematic investigation of the results of the trial and error tests, eventually led to the use of two alternative reactor systems for maintaining reducing conditions.

The serum vial is very simple and inexpensive, but is dependent upon the choice of septum material for its usefulness. Butyl rubber septa maintain reducing conditions longer than other septum materials, but even this utility is compromised by the process of sampling the reaction. It was presumed that diffusion of oxygen through septum punctures contributed to the loss of reducing conditions, and that the syringe needles themselves could carry oxygen into the vessel. If the serum vial was not pressurized, then a partial vacuum induced by removing samples could draw air in through septum

punctures. The two-chambered reactor, or TCR, was developed to avoid these problems.

In the TCR, any oxygen which may perfuse through septum wounds is constantly purged with purified gas. The purging stream is prevented from convectively carrying oxygen to the reaction mixture by a narrow constriction. Samples are removed by a capillary, one end of which always remains inside the reactor. No matter how many samples are taken, no vacuum is ever induced in the TCR. Kinetic studies were performed with the TCR.

The third reactor system for conducting reductive dechlorination reactions is the hermetically-sealed glass ampoule. By sealing in glass, reactants can be in contact for long periods of time, perhaps indefinitely. Reductive dechlorination of the trichlorophenols required up to several weeks to be observed, and the ampoule method proved quite useful in these studies.

With the arsenal of reactor and analytical systems in hand, the objectives could be pursued. All of the chlorophenols tried were reductively dechlorinated to some extent by vitamin B_{12s}. By combining the data from the individual chlorophenols, a pathway was constructed for the sequential reductive dechlorination of the family of chlorophenols (Chapter 6). Once the pathway was constructed, it could be seen that the regiospecificity of vitamin B_{12s} reductive dechlorination of chlorophenols varied significantly from that of microbial consortia. It was proposed that, if vitamin B₁₂ does play a role in the reductive dechlorination of chlorinated phenols by the microbial consortia, then it must be in conjunction with the directing influence of apoenzymes in the cells.

In addition to meeting the objective of establishing the dechlorination pathway, a mechanism was proposed to account for the pathway observed. Careful examination of the regiospecificity, and consideration of precedent chemistry, led to the hypothesis of

nucleophilic aromatic substitution by the nucleophile vitamin B_{12s}, followed by reductive cleavage of the aryl cobalamin to form the product. To my knowledge, this is the first description of the participation of vitamin B₁₂ in nucleophilic aromatic substitution.

The kinetics of the experimental system provided a special challenge. In order to accelerate the reaction, high concentrations of vitamin B₁₂ were used. Inconsistent results were obtained, with different rates occurring under seemingly identical reaction conditions. Often the progress of the reaction started slowly, then proceeded more quickly. Such kinetics are typical of autocatalytic mechanisms, and models of such a system were considered. The autocatalytic model described the PCP progress curve if it was assumed that the species responsible for autocatalysis, the tetrachloroaryl-cobalamin, would decay to cobalamin and TeCP during the analytical workup. The progress curve also resembled the complement to the third component of a sequence of first-order reactions. This could occur if it were assumed that the σ -complex intermediate of nucleophilic aromatic substitution could decay back to original substrate, and be detected in the chlorophenol assay as such. Both the autocatalytic model and the artifact models described the shape of the PCP progress curves. Ultimately, a simple pseudo-first order model predicted the extent of reaction, if not the shape of the reaction curve, as well as did the more intricate models.

Not only does the regiospecificity of vitamin B_{12s} reductive dechlorination of phenols differ from that of anaerobic bacteria, but the rates are considerably slower.

The effect of E_H was studied by using a milder reducing agent, DTT. Repeated attempts to support reductive dechlorination with DTT met with failure, using the same conditions as used with Ti(III) citrate. Addition of 50% dioxane, a condition suggested by successful reductive dechlorination of an aromatic substrate elsewhere (Assaf-Anid et al., 1992) did not alter this result. Reductive dechlorination of pentachlorophenol by

vitamin B₁₂ requires reducing conditions sufficient to reduce the vitamin to vitamin B₁₂s.

Engineering Implications

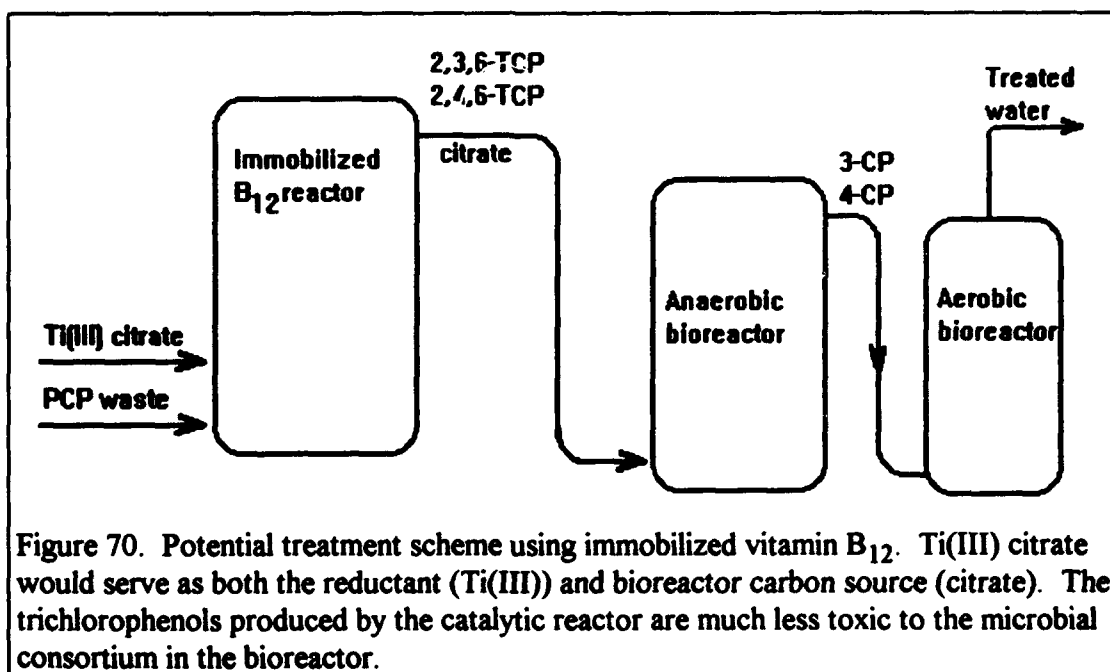
The original impetus for this research was the idea that reductive dechlorination could be accomplished catalytically, rather than biologically. There would be several advantages to this approach. One of the limitations to biological treatment is the production, care and nourishment of the bacteria responsible for the process. Catalytic reduction would require no time for biomass enrichment, no acclimation time, and would not be sensitive to potentially toxic levels of substrate. Vitamin B₁₂ can be immobilized, using some of the same methods used to immobilize enzymes to solid substrates. An immobilized vitamin B₁₂ reactor could be used to treat contaminated groundwater that is too heavily polluted for a bioreactor.

Unacclimated anaerobic consortia preferentially produce 3,4,5-TCP from PCP (Nicholson, 1992). In contrast, this research shows that vitamin B₁₂ preferentially removes the *ortho* and *para* chlorines, and can easily reductively dechlorinate PCP at least to a mixture of 2,3,6-TCP and 2,4,6-TCP. 3,4,5-TCP is approximately ten times more toxic to dechlorinating bacteria than is 2,4,6-TCP (Madsen and Aamand, 1992).

A reasonable scheme would treat groundwater that is too highly polluted for bacterial treatment to a chlorophenol level that is tolerated by the biological reactor. After five days in Experiment 314, only 4% of the original concentration of PCP remained. The rest was converted to a mixture of 2,3,4,6- and 2,3,5,6-TeCP and 2,4,6- and 2,3,6-TCP (Chapter 6). This process would circumvent the production of the toxic 3,4,5-TCP. The 2,3,6-TCP and 2,4,6-TCP could then be dechlorinated to 3-CP or 4-CP by anaerobic microbial consortia (Woods et al., 1989). These monochlorophenols can then be mineralized by a final aerobic bioreactor.

Such a process would require reduction of the catalyst to the vitamin B_{12s} state, requiring an E_H of at least -0.61 V. Ti(III) citrate could be added to the feed to reduce the immobilized vitamin B₁₂. The citrate would feed the subsequent anaerobic bioreactor, and the oxidized Ti(IV) would precipitate as environmentally inert TiO₂. The process is shown schematically in Figure 70.

This process as envisioned would potentially be subject to severe environmental constraints. The laboratory process was well controlled with respect to redox species, an unlikely condition for real groundwater. The process is also extremely sensitive to O₂, which would have to be rigorously excluded. Intermediate-depth groundwaters with high Fe²⁺ content would be especially difficult to treat, as these waters contain high levels of oxidized species (Snoeyink and Jenkins, 1980). The high demand for reductant may be met by a combination reactor which would include an anaerobic microbial consortium and immobilized vitamin B₁₂, along with auxiliary reductant. The microbial consortium would provide the bulk of the reducing power needed, with Ti(III) citrate added to reduce the immobilized vitamin B₁₂.



Suggestions for Future Research

The research reported herein suggests a myriad of additional research, both in applied engineering and basic chemistry. I have uncovered many more questions than answers.

From the environmental engineering perspective, the use of immobilized vitamin B₁₂ as a treatment process should be pursued. Preliminary results have shown that vitamin B₁₂ immobilized on agarose beads (Sigma Chemical Company, St. Louis, MO) will reductively dechlorinate PCP. These results were beyond the scope of this thesis and were not pursued nor reported here, but the concept deserves investigation. Immobilization means that the catalyst can potentially be used for an extended time. Recent advances in fermentative production of vitamin B₁₂ may make the catalyst readily available at reasonable cost (Mazumder et al., 1987).

While vitamin B₁₂ exhibits a different regiospecificity than bacteria in its reductive dechlorination of the chlorophenols, it is by no means clear that it plays no role *in vivo*. Its natural ability to reductively dechlorinate chlorophenols may indeed be responsible for biological reductive dechlorination, in conjunction with apoenzymes which direct the regiospecificity. Attention should be paid to the corrinoid or cobalt nutritional requirements of microbial consortia which are cultivated for reductive dechlorination duty.

The threefold variability of the kinetics of PCP reductive dechlorination is frustrating. Whatever factor is responsible for the variability may indicate methods to accelerate the reaction. Temperature will most likely be found to be responsible, but the variable amounts of methanol in which the PCP was dissolved, added with the substrate, may also be a factor.

Many features of basic chemistry arising from this study present future research challenges. The vast majority of the scientific literature on nucleophilic aromatic substitution is dedicated to aromatic nitrates. The nature of the cobalamin σ -complexes and the arylcobalamins formed during the course of this reaction may also prove interesting. Of particular intrigue is the reductive dechlorination, albeit slow, of 2,4,6-TCP. Formation of the σ -complex at any of the chlorinated carbons would be at a nonactivated site. Initial attack at a nonchlorinated carbon would require subsequent migration of the cobalamin to the adjacent carbon. Migration of a nucleophile to the 1 position after initial attack at the 3 position is known (Servis, 1965, 1967). It is also fascinating that this substrate was the only one for which dechlorination at the 2 position was observed.

Additional experiments should be undertaken to discern whether an autocatalytic mechanism is responsible for the shape of the PCP progress curve. Since both the autocatalytic model and the artifact model required that arylcobalamins decompose during analytical workup, investigation as to the stability of the σ -complex and the arylcobalamin would prove fruitful. Experiments with aliphatic substrates may shed light on the autocatalytic mechanism, since nucleophilic substitution with aliphatic halides involves no intermediate comparable to the σ -complex.

The year of this dissertation (1993) marks the 45th anniversary of the isolation of vitamin B₁₂ in crystalline form. I have been humbled in my literature search by the depth and breadth of research that has preceded my efforts. After 45 years of intensive study by some of the preeminent scientists of the world, I am proud to in some small way add to the sum of knowledge about this marvelous molecule. Many of its secrets have been unveiled, but it promises many more challenges and opportunities in the future.

Chapter 10

Conclusions

Vitamin B_{12s} (cob(I)alamin) is extremely sensitive to O₂, being immediately oxidized to vitamin B_{12a} (hydroxycob(III)alamin) (Pratt, 1972). In order to study the reactions of vitamin B_{12s}, the reactants must be protected from atmospheric oxygen. This may be accomplished by using serum vials with butyl rubber septa, hermetically-sealed glass ampoules, or a new reactor design, the two-chambered reactor (TCR). The TCR consists of a glass vessel with an upper and lower chamber separated by a narrow neck. The reacting solution in the lower chamber is protected from oxygen by purging the upper chamber with purified inert gas. Serum vials may maintain reducing conditions for several days, but sampling of the contents introduces oxygen which diminishes the effectiveness of the seal. Ampoules can maintain reducing conditions for many months, allowing the study of relatively slow reactions. The TCR can maintain reducing conditions for several days, and multiple samples of the reaction solution may be taken without diminishing the effectiveness of the vessel to maintain reducing conditions.

Vitamin B₁₂ catalyzes the reductive dechlorination of chlorophenols when reduced to its nucleophilic, vitamin B_{12s} state. The less reduced vitamin B_{12r} form is inactive. The reducing potential of the medium therefore must be sufficient to fully reduce the cobalamin. Reductive dechlorination occurs with a regiospecificity consistent with classical nucleophilic aromatic substitution by the nucleophile on the ring to form first a σ -complex, then an arylcobalamin. Reductive cleavage of the carbon-cobalt bond results in the dechlorinated arene.

The regiospecificity of vitamin B_{12s}-catalyzed reductive dechlorination of chlorophenols is unlike that expressed by anaerobic microbial consortia. Whereas

unacclimated consortia preferentially dechlorinate pentachlorophenol at the *ortho* position, vitamin B_{12s} attacks the *meta* and *para* chlorines. This pattern with pentachlorophenol more closely resembles that seen with acclimated consortia, but even these bacteria preferentially remove *ortho* chlorines from lesser-chlorinated congeners, while these same positions continue to resist vitamin B_{12s} attack.

Exposure of the reaction medium to white light does not significantly increase the overall rate of reaction. Light over a broad spectrum is known to induce homolytic cleavage of organocobalamins, forming free radicals (Schrauzer *et al.*, 1968). The low sensitivity of the rate to light indicates that the rate-limiting step(s) is a process other than homolytic cleavage.

The kinetics of reductive dechlorination of pentachlorophenol by vitamin B_{12s} follows first order kinetics, with some anomalies. A threefold variability was found in measured pseudo-first order kinetic constants, ranging from 0.06 hr⁻¹ to 0.2 hr⁻¹. This variability was associated with agitation of the reaction, but may have been due to temperature of the reacting solution. In addition, several experiments demonstrated a lag period early in the reaction. The lag could be explained as an autocatalytic process if it was assumed that the autocatalytic species could decompose to the dechlorination product upon sample workup. Individual experiments could also be modeled as the result of an alternative artifact of analysis, whereby the starting pentachlorophenol and the first intermediate, the σ -complex, are both detected as pentachlorophenol. Although these models were internally consistent within individual experiments, they still did not explain the threefold variability of rates. A simple first order model, while not descriptive of the shape of the reaction progress curve, did predict the extent of reaction.

Both regiospecificity and kinetic considerations indicate that the reductive dechlorination of the chlorophenols by anaerobic microbial consortia is not a

consequence of the casual presence of reduced vitamin B₁₂ in the medium. The dechlorination pattern exhibited by vitamin B₁₂ is significantly different from that of both acclimated and unacclimated microbial consortia. The observed rates of reductive dechlorination of pentachlorophenol by vitamin B₁₂s were attained by using high concentrations of vitamin B₁₂. These rates were compared with rates of reductive dechlorination of pentachlorophenol by acclimated anaerobic microbial consortia. Vitamin B₁₂ concentrations used in this study exceed that found in natural systems by at least two orders of magnitude. The estimated corrinoid content of the microbial consortium is insufficient to explain the rate of reductive dechlorination as an abiotic vitamin B₁₂ reaction. If corrinoids participate in biological reductive dechlorination, they probably do so in concert with specific apoenzymes which direct the specific activity to that which is observed.

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APPENDICES

Appendix A

Chlorophenol Assay Procedure

The chlorophenol assay procedure used in this research was developed as a miniaturization of the acetylation procedure of Voss et al. (1980). The original procedure was designed to detect minute quantities of various chlorinated pollutants in pulp mill wastes, and called for a sample size of 50 mL. Reactions were conducted in separatory funnels, which were shaken by hand for part of the reaction and extraction stages. Separatory funnels are expensive and difficult to clean, the procedure was tedious and time-consuming, and the analyst risked repetitive motion trauma from manipulating the funnels (the author suffers from carpal tunnel syndrome). In the present research, I have used relatively higher concentrations of chlorophenols, so a lower volume procedure using smaller glassware was investigated.

The method is similar to a method developed by Perkins (1992). In the present method, the internal standard and K_2CO_3 are combined in a single reagent, and the reaction mixture is agitated throughout the acetylation and extraction stages. In the Perkins method, internal standard and K_2CO_3 are added separately and the reaction vessels are stationary during the two stages.

The use of Viton closures on the autosampler vials is necessary to minimize extraneous negative peaks in the resulting chromatograms. Even with Viton, extraneous peaks interfere with chromatographic analysis, but the performance of Viton is superior to other closure septum material.

Equipment:

100 μ L syringe

3 mL fixed volume pipette

1 mL pipette (volumetric or repeating)

100 μ L pipette

10 mL acid washed* screw-top culture tubes with Teflon-lined caps

Autosampler vials with Viton closures

Pasteur pipettes and bulb

Reagents:

Acetic anhydride, reagent grade

Hexane, HPLC grade

Chlorophenol reagent (aqueous):

43 g/L K_2CO_3

1 mg/L 2,4,6-tribromophenol (TBP)

Procedure:

1. Add 1.0 mL chlorophenol reagent to culture tube
2. Add 100 μ L sample
3. Add 100 μ L acetic anhydride
4. Shake on wrist-action shaker 20 minutes
5. Add 3.0 mL hexane
6. Shake 10 minutes
7. With a new pasteur pipette, transfer hexane fraction to autosampler vials; seal with Viton caps
8. Place samples in autosampler carrel for analysis, or save for hand injection.

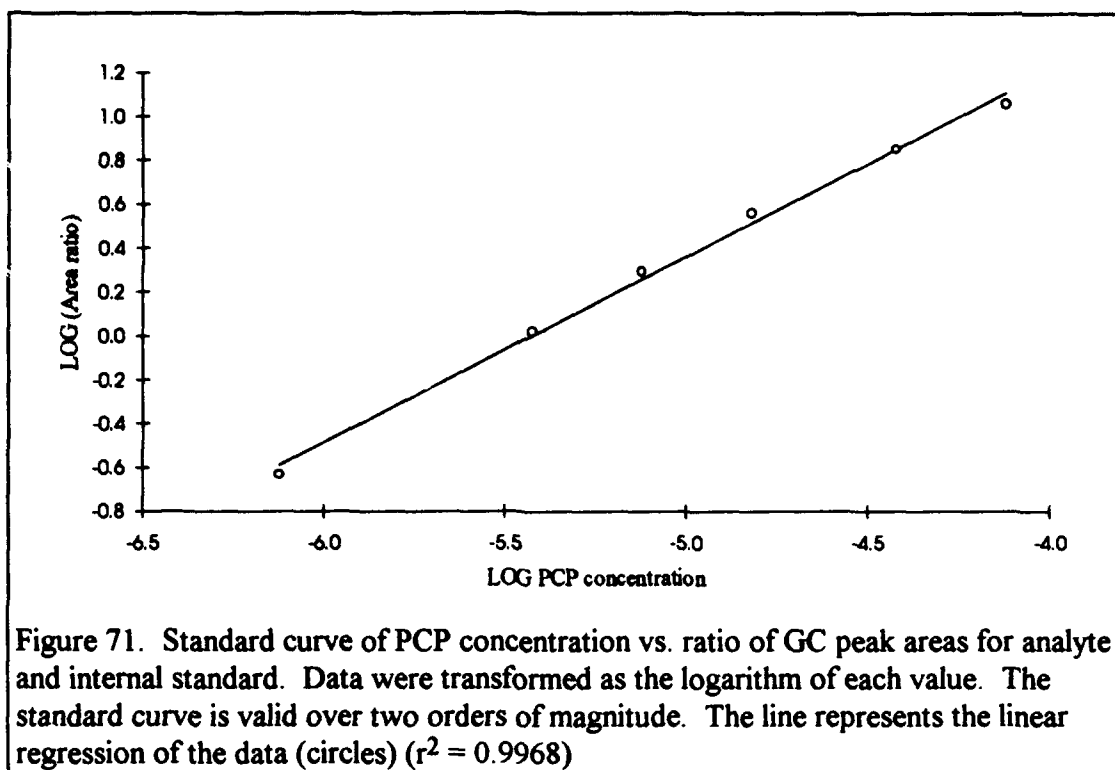
GC Analysis: Temperature program: Initial temp 45°C for 2 minutes; ramp 15°/min to 105°; ramp A 5°/min to 215°, hold 5 minutes.

* Soak at least overnight in 50% v/v H_2SO_4 , then rinse three times with tap water and three times with distilled, deionized water.

Table 10 shows typical elution times and retention times relative to the internal standard (2,4,6-tribromophenol). The actual elution times are dependent upon the tuning of the carrier gas flow, and also vary from column to column, but the relative retention times (RRT) are diagnostic of the particular chlorophenol.

Table 10. Relative GC elution times for chlorophenol metabolites. RRT is retention time relative to 2,4,6-tribromophenol.		
Congener	Typical retention time	RRT
2-CP	13.448	0.504
3-CP	14.061	0.527
4-CP	14.302	0.536
2,6-DCP	16.664	0.624
2,5-DCP	17.164	0.643
2,4-DCP	17.164	0.643
3,5-DCP	17.489	0.656
2,3-DCP	17.983	0.674
3,4-DCP	18.622	0.698
2,4,6-TCP	19.632	0.736
2,3,6-TCP	20.765	0.778
2,3,5-TCP	20.975	0.786
2,4,5-TCP	21.119	0.792
2,3,4-TCP	22.237	0.833
3,4,5-TCP	22.580	0.846
2,3,5,6-TeCP	24.218	0.908
2,3,4,6-TeCP	24.322	0.912
2,3,4,5-TeCP	25.760	0.965
2,4,6-TBP	26.682	1.000
PCP	28.675	1.075

The data are analyzed as the ratio of peak area of the analyte to the area of the internal standard. If the data are transformed in a log-log fashion, they are linear over two orders of magnitude. Figure 71 shows the plot of the common logarithm of [PCP area/TBP area] vs log(time).



Appendix B

Preparation of Ti(III) Citrate Solution

A stock solution of 250 mM Ti(III) citrate in 0.66 M Tris buffer was prepared by a modification of the method of Zehnder and Wuhrman (1976). In the original method, the HCl in which the commercial TiCl_3 is dissolved is neutralized with a fairly large volume of saturated sodium carbonate solution. The resulting solution is about 50 mM Ti(III) citrate. The more highly concentrated stock allows for reduction of vitamin B_{12} solutions with the addition of smaller volumes of reductant. The newly-developed preparation also standardizes the concentration of stock solution.

Thirty mL of 13% TiCl_3 in 20% HCl (Fluka Chemical Corp., Ronkonkoma, NY) was added to a slurry of 8.0 g Tris and 14.7 g Na_3 citrate (Mallinckrodt, Inc, St. Louis, MO) in about 40 mL distilled, deionized water. During preparation, the solution was kept in an ice bath and was constantly sparged with argon or nitrogen. The pH was adjusted to 8.2 by the addition of flakes of solid NaOH. The ice bath was necessary because of the generation of heat as the solid NaOH was added to the acid solution. The resulting Ti(III) citrate solution was then diluted to 100 mL with distilled, deionized water. This stock solution was divided into 15 mL aliquots and stored frozen in serum vials capped with Teflon-lined rubber septa and aluminum crimp seals. The frozen stock solutions maintained potency for at least five months.

Appendix C

Reductive Dechlorination Reactions by the Glass Ampoule Method

Many of the reactions in this research were conducted in hermetically-sealed glass ampoules. Some skill is required to achieve a lasting seal on these ampoules and thereby ensure the longevity of the vitamin B₁₂s.

The best results were obtained when the vitamin B₁₂ and chlorophenol solutions were first introduced to the ampoule, and then the neck of the vessel was drawn out in a bunsen burner flame to a narrow tube. The contents were sparged for about five minutes with inert gas through a capillary inserted into the neck, after which the reductant was introduced. The ampoule could then be quickly sealed with the bunsen burner, as the glass in the drawn-out neck melts much more quickly than the neck in its original condition. The best seals were obtained by holding both ends of the neck and allowing the neck glass to melt together and split naturally, without further drawing out the glass.

Appendix D

Multiple linear regression of kinetic data

Data from multiple experiments can be compared to each other by use of multiple linear regression. Any variable can be analyzed. Discrete variables, that is, variables which cannot be quantified with a value along a continuum, can be analyzed in this way. Treatments, such as type of reactor, can be analyzed with indicator variables.

Table 11 shows the rate data for the reductive dechlorination of PCP performed with identical reactant concentrations in ampoules, in a serum vial with butyl rubber stopper, or in a TCR with or without illumination from a source of white light.

Table 11. Multiple linear regression of rate data from experiment 330.					
Reactor	Time (hours)	"x1"	"x2"	"x3"	Transformed data
Ampoule	0	0	0	0	0
Ampoule	18	0	0	0	1.24
Ampoule	25	0	0	0	1.87
Ampoule	46	0	0	0	2.68
Ampoule	71.5	0	0	0	4.37
TCR dark	0	0	0	0	0
TCR dark	7	7	0	0	0.58
TCR dark	21	21	0	0	1.83
TCR dark	31	31	0	0	2.90
TCR dark	45	45	0	0	4.07
TCR light	0	0	0	0	0
TCR light	7	0	7	0	0.66
TCR light	21	0	21	0	1.89
TCR light	31	0	31	0	2.80
TCR light	45	0	45	0	4.29
Vial	0	0	0	0	0
Vial	7	0	0	7	0.45
Vial	21	0	0	21	1.37
Vial	31	0	0	31	2.13
Vial	45	0	0	45	2.63

The "Reactor" column is simply an identifier and is not used in the regression. "Time" is the time, in hours, of each sample. The indicator variables x_1 , x_2 , and x_3 are explained in the model, below. A pseudo-first-order kinetic model was assumed. The last column is the PCP concentration data transformed as is appropriate for a first order kinetic model, i.e., the negative natural logarithm of the ratio of the PCP concentration to initial PCP concentration (Levenspiel, 1972).

The regression model is

$$y = \beta_0 + (\beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4) t$$

where y is the predicted value of the transformed regression data, β_0 is the intercept of regression, t is time, β_4 is the slope of the line due to reaction in the ampoule, β_1 and β_2 are additional contributions to the slope due to reaction in the dark or illuminated TCR, respectively, β_3 is the additional contribution to the slope by the vial, and x_1 , x_2 , and x_3 are indicator variables which activate the additional contributions to the slope by the TCRs or the vial. The indicator variables can be only 1 or 0. When $x_1 = x_2 = x_3 = 0$, then the data refer only to the ampoule. If $x_1 = 1$ and $x_2 = x_3 = 0$, then the data refer only to the dark TCR. If $x_2 = 1$ and $x_1 = x_3 = 0$, then the data refer only to the illuminated TCR, and when $x_3 = 1$ and $x_1 = x_2 = 0$, then the data refer only to the vial.

The data was subjected to multiple linear regression using Excel® spreadsheet software (Microsoft Corporation, Redmond, WA). The coefficients of regression were as shown in Table 12.

Table 12. Statistical data for the multiple linear regression of rate data from experiment 330.

Coefficient	Value	Standard error (se) value for the coefficient
β_0 (intercept)	0.0136	0.0441
β_4 (ampoule)	0.0615	0.00157
β_3 (dark TCR)	0.0288	0.00245
β_1 (light TCR)	0.0312	0.00245
β_2 (vial)	0.00539	0.00351
degrees of freedom	14	
r^2	0.994954	
se of estimated y values	0.119	
F-statistic	690	
regression sum of squares	38.9	
residual sum of squares	0.197	

The coefficient $\beta_4 = 0.0615$ is the slope of the line of the transformed concentration data vs time, or the kinetic constant, k , of the first order reaction occurring in the ampoules. The kinetic constant for reaction in the vial was $\beta_4 + \beta_2 = 0.067$. The reactions in the TCR were significantly faster, $\beta_4 + \beta_1 = 0.0903$ for the dark TCR and $\beta_4 + \beta_2 = 0.0927$ for the illuminated TCR.

The statistical significance of each parameter can be determined from its t -statistic, t^* , or the ratio of its value to its standard error. The t^* can then be compared to standard statistical distribution values to determine the probability that that value derives from more than mere chance. From this evaluation it can be seen that the difference in reaction rates between the ampoule and the vial was statistically significant only at the 85% probability level ($p = 0.15$), while the faster rate in the TCRs was statistically significant ($p < 0.0001$). Statistical comparisons must be made against the standard variable, in this case β_4 . The data may be rearranged to compare the illuminated to the dark TCR, so that one of the TCR data sets correspond to β_4 .

Table 13. Significance of difference in rates of vial and TCR reactions compared to the ampoule.		
k contribution due to. . .	t*	p =
Ampoule	39.13345	
TCR dark	11.76997	<0.0001
TCR light	12.75939	<0.0001
vial	1.53698	0.15

Appendix E

Raw Data

Table 14. Data for chapter six figures.

log-log standard curves		
	slope	intercept
PCP	0.8470453	4.5973866
2345	0.8372048	4.3561713
2346	0.8408908	4.4177628
2356	0.8405844	4.3500423
234	0.8142948	4.049835
235	0.7907639	3.8497862
236	0.8311957	4.031036
245	0.7291007	3.6190729
246	0.8210607	4.0433166
345	0.821059	4.0316686
23	0.7679372	3.3013095
24	0.8054895	3.3810792
25	0.6983963	2.9432342
26	0.6950063	3.0169954
34	0.6986411	2.9369317
35	0.7300158	3.1658956

3.94

Conversion of 2,3,4,6- TeCP

Time (Days)	Ratios			Concentrat ions			Total
	TeCP	2,4,6	2,3,6	TeCP	2,4,6-TCP	2,3,6	
0	0.7471116		0	3.9423763		0	3.9423763
14	0.4200558	0.1427256		1.9877463	1.1110486		0 3.0987949
31		0 0.3561502	0.0556853		0 3.3838726	0.4378597	3.8217323

Ti(III) control 31 days

0	0.7471116	3.9423763
31	0.7350857	3.8670259

Table 14, continued.

Conversion of 2,3,5,6-
TeCP

Time (Days)	Ratio 2,3,5,6- TeCP	Ratio 2,3,6-TCP	Concentration 2,3,5,6- TeCP	Concentration 2,3,6-TCP	Total
0	1.4548334	0	10.439241	0	10.439241
14	0.9650946	0.2617247	6.4065163	2.8179541	9.2244704
Ti control					
14 days					
0	1.4548334		10.439241		
14	1.4345949		10.266706		

Conversion of 2,3,4,5-
TeCP

Time	Ratio 2345	Ratio 2,4,5	Ratio 2,3,5	Conc. 2,3,4,5- TeCP	Conc. 2,4,5	Conc. 2,3,5	Total
0	0.8475649	0	0	5.1401247	0	0	5.1401247
8	0.4465115	0.2262514	0.0480486	2.3906163	1.415921	0.2913498	4.0978871
22	0	0.3879845	0.0809851	0	2.9667996	0.5638061	3.5306058

control 31
days

0	0.8475649	5.1401247
22	0.8358263	5.0552074

Conversion of
Trichlorophenols

Time (Days)	Ratio 2,4,6-TCP	Ratio 2,6-DCP	Ratio 2,4-DCP	Conc. 2,4,6-TCP	Conc. 2,6-DCP	Conc. 2,4-DCP	Total
0	7.4907811	0		138.23404	0		138.23404
3	7.1712031	0		131.08508	0		131.08508
9	6.2166776	0.0928463		110.15398	1.492181		111.64616
14	6.6308398	0.1097024		119.1557	1.8970005		121.0527
31	6.4886702	0.1347758		116.05146	2.5508989	0	118.60235
60	4.9746266	0.2115609	0.2115609	83.966592	4.8803512	9.2256104	98.072554

Ti control
41 days

0	7.4907811	138.23404
60	7.5534705	139.64431

Table 14, continued.

	Ratio	Ratio	Conc.	Conc.		
	2,3,6-TCP	2,6-TCP	2,3,6-TCP	2,6-TCP	Total	
0	7.3457958		0 155.68116		0 155.68116	
2	6.6652991		0 138.49775		0 138.49775	
8	6.4638219		0 133.47664		0 133.47664	
22	7.0613466		0 148.45729		0 148.45729	
31	6.33148	0.0723021	130.19568	1.0412242	131.2369	
ti control						
41 days						
0	7.3457958		155.68116			
31	6.9491794		145.62478			
	Ratio	Ratio	Ratio	Conc.	Conc.	Conc.
	2,3,4-TCP	2,3-DCP	2,4-DCP	2,3,4-TCP	2,3-DCP	2,4-DCP
0	3.4210598			48.145258		
2	3.2128084			44.571499		
8	2.9529919	0.1095212	0.0435385	40.186729	2.8204101	1.2961077
22	2.4271664		0.3445201	31.586248		16.9011
				0.3439385		
						1.0071054
Ti						
control 41d						
0	3.4210598			48.145258		
22	3.1410449			43.352003		
	Ratio	Ratio	Ratio	Conc.	Conc.	Conc.
	3,4,5-TCP	3,4-DCP	3,5-DCP	3,4,5-TCP	3,4-DCP	3,5-DCP
0	5.8403756			105.47533		
8	4.9577553	0.0434371	0.0831644	86.394799	0.7023019	1.5266697
22	4.536439	0.3886109	0.2310476	77.537477	16.168676	6.1890995
Ti Control						
0	5.8403756			105.47533		
22	5.908389			106.97322		
Actually 41 days						
2,4,5-TCP	Ratio	Ratio	Conc.			
	245	24,25	2,4,5			
0	0.7101542		6.797877			
5	0.6749781	0.1623974	6.3403433			
31	0.4891853	0.2743345	4.0770626			

Table 14, continued.

2,3,5

	Ratio 2,3,5-TCP	Ratio 2,5-DCP	Ratio 2,3-DCP	Conc. 2,3,5-TCP	Conc. 2,5-DCP	Conc. 2,3-DCP	TOTAL
0	7.944787			186.12496			186.12496
8	7.2573444	0.1199675		165.99695	2.9314392		168.92839
11	6.9187663	0.1509204		156.26469	4.0720567		160.33674
14	6.7218	0.3408767		150.66031	13.075967		163.73628
31	4.3013447	1.4036453		85.667588	99.214659		184.88225
59	1.9520942	1.1042646	0.1550131	31.544809	70.371934	4.4337808	106.35052

Ti control

40 days

0	7.944787	186.12496
40	8.0144391	188.19087

Table 15. Data for experiment 249

Standard curves from log-log regression of mhs250_2 and mhs2502B

pcp 0.96512 8.10848

2356 0.9653 7.91145

2346 1.01251 8.28328

Time (hr)	Areas		Concentrations						
	TBP	PCP	2346	2356 PCP					
0	4514787	280887		2.2E-06					2.2E-06
0	4303085	258690		2.2E-06					2.2E-06
0	4612157	283209		2.2E-06					2.2E-06
1	4426150	231007		1.9E-06					1.9E-06
1	4495680	259300		2.1E-06					2.1E-06
1	4389008	254513		2.1E-06					2.1E-06
2	4389069	223356		1.8E-06					1.8E-06
2	4601827	252835		2E-06					2E-06
2	4479072	243543		1.9E-06					1.9E-06
5.66	4396170	119745	48665	40420	9.5E-07	7.7E-07	4.9E-07	1.3E-06	2.2E-06
5.66	4456483	137430	51040	40821	1.1E-06	8E-07	4.9E-07	1.3E-06	2.4E-06
5.66	4540682	138821	53304	42522	1.1E-06	8.2E-07	5E-07	1.3E-06	2.4E-06
10.66	4336650	54621	79655	63673	4.3E-07	1.3E-06	8E-07	2.1E-06	2.5E-06
10.66	4530003	55711	81913	67444	4.2E-07	1.3E-06	8.2E-07	2.1E-06	2.5E-06
10.66	4424861	52092	81464	66107	4E-07	1.3E-06	8.2E-07	2.1E-06	2.5E-06
19	4675853	11357	102428	83564	7.8E-08	1.5E-06	9.8E-07	2.5E-06	2.6E-06
19	4567514	9825	98742	81843	6.8E-08	1.5E-06	9.9E-07	2.5E-06	2.6E-06
19	4606589	13910	100997	83149	9.7E-08	1.5E-06	1E-06	2.5E-06	2.6E-06

Table 16. Data for experiment 306.

Experiment 306

log-log standard curves

PCP	0.847045	4.597387
2345	0.837205	4.356171
2346	0.840891	4.417763

First Sample

Hrs	Areas				Conc. ($\mu\text{mol/L}$)				
	TBP	PCP	2346	2356	PCP1	2346	2356	TeCP	Total CP
0	419931	352043			3.034114				3.034114
1	436572	332115			2.705386				2.705386
2	414161	289508	32251	17942	2.448237	0.267877	0.147358	0.415235	3.278707
3	416572	231271	50679	25331	1.865197	0.455368	0.220938	0.676306	3.217809
4	436071	165763	60841	30633	1.192668	0.535947	0.262498	0.798445	2.789559
5.25	447528	176004	92209	49120	1.241535	0.852065	0.44732	1.299385	3.840304
6	427465	133933	99265	49789	0.949332	0.982298	0.480207	1.462506	3.874343
7	425343	99113	109781	56436	0.66927	1.113831	0.561068	1.674899	4.019068

Second Sample

Hrs	TBP	PCP	2346	2356					
0	TBP	PCP	2346	2356					
1	413726	348034			3.046437				3.046437
2	404632	320489			2.837352				2.837352
3	421165	291795	32852	16533	2.422644	0.268417	0.130993	0.39941	3.221464
4	414139	235097	49449	27478	1.914877	0.445346	0.245195	0.690542	3.29596
5.25	425811	173830	65949	30361	1.29745	0.606814	0.267209	0.874023	3.045496
6	409953	162835	89728	43220	1.256171	0.915543	0.426315	1.341857	3.939886
7	422200	126426	98255	52646	0.899907	0.984832	0.520956	1.505789	3.911484
	427001	98865	108928	56694	0.664235	1.098453	0.561518	1.65997	3.984176

Table 17. Data for experiment 314.

Experiment 314

log-log standard curves

PCP	0.84705	4.59739
2346	0.84089	4.41776
2356	0.84058	4.35004
236	0.8312	4.03104
246	0.82106	4.04332

Calculated initial concentration of PCP 4.27 $\mu\text{mol/L}$

Sample 1

Hours	Areas				Conc.		
	TCP	PCP	2346	2356	PCP	2346	2356
0	350582	540884			6.23393		
0.5	388460	443135			4.36533		
1	378883	420827			4.22937		
1.5	381175	413608			4.11445		
2	382117	366107			3.5522		
3	371791	375307			3.77806		
4	373925	366458			3.64839		
5	382051	357502			3.45455		
6	357255	296382			2.99686		
7	381028	312143			2.95258		
9	421328	286837	82693	59449	2.37304	0.80422	0.65045
11	362164	205489	101521	52594	1.91374	1.22876	0.67312
14	378262	162967	129051	65208	1.38266	1.55213	0.82545
19	378320	94670	200016	72829	0.72804	2.61313	0.94128
24	376120	73522	213873	81830	0.5439	2.84951	1.08878
120							

Sample 2

Hours							
0	377089	499029			5.20113		
0.5	377408	402281			4.02872		
1	377660	389197			3.87143		
1.5	361406	353062			3.63468		
2	374079	382890			3.84043		
3	369720	368995			3.72766		
4	359364	339087			3.48871		
5	380507	343698			3.31344		
6	348823	292322			3.03278		
7	388315	333793	90628	34154	3.12515	0.98819	0.37069
9	378172	276612	87981	46686	2.58285	0.98446	0.55485
11	392702	223577	87626	45162	1.92146	0.93678	0.50998
14	399362	164711	177139	69440	1.31323	2.12068	0.83394
19	401322	101000	205934	77268	0.73295	2.52195	0.94144
24	393979	67049	230172	84305	0.46183	2.94268	1.0675

Table 17, continued.

Average					120 hours	Area	Conc ($\mu\text{mol/L}$)
Hours	PCP	2346	2356	Total	TBP	395447	
0.5	4.19702			4.19702	PCP	27626	0.16142
1	4.0504			4.0504	2346	81645	0.85414
1.5	3.87457			3.87457	2356	40823	0.4485
2	3.69632			3.69632	2,3,6-TCP	64481	1.59477
3	3.75286			3.75286	2,4,6-TCP	61662	1.23747
4	3.56855			3.56855			
5	3.38399			3.38399			
6	3.01482			3.01482			
7	3.03886	0.98819	0.37069	4.39774			
9	2.47794	0.89434	0.60265	3.97494			
11	1.9176	1.08277	0.59155	3.59192			
14	1.34795	1.83641	0.8297	4.01405			
19	0.7305	2.56754	0.94136	4.2394			
24	0.50287	2.89609	1.07814	4.4771			

Table 18. Data for experiments 330.

Experiments	log-log data	slope	int
330	PCP	0.9120117	4.8943636
	2,3,4,6	0.8408908	4.4177628
	2,3,5,6	0.8405844	4.3500423
	2,3,6	0.8311957	4.031036
	2,4,6	0.8210607	4.0433166
	2,4	0.8054895	3.3810792
	2,6	0.6950063	3.0169954
	2,3,5	0.7907639	3.8497862
	3,4,5	0.821059	4.0316686
	2,3	0.7679372	3.3013095

Ampoule	Ratios	Concentratio ns
Time (hours)	Ratio	Conc. ($\mu\text{mol/L}$)

0	2.4492059	11.481528
18	0.7878781	3.310641
25	0.4424037	1.7582909
46	0.212018	0.78492
71.5	0.0454105	0.1448923

Time	Ratios			Conc. ($\mu\text{mol/L}$)		
	Dark TCR	Light TCR	Vial	Dark	Light	Vial
0	2.1502645	2.1465769	1.8302193	9.954331795	9.9356153	8.3420163
7	1.269585	1.1794828	1.21578	5.586054006	5.1528868	5.327015
21	0.4066076	0.3832797	0.5251092	1.60292161	1.5023701	2.1217905
31	0.1530358	0.1629531	0.2624748	0.549018148	0.6084294	0.9919397
45	0.0525718	0.0417345	0.1663237	0.170128491	0.1366376	0.601501

Ti(III) Control reaction, p
337

Time (hours)	TBP	PCP	Ratio	Conc (mol/L)
0	694117	1276364	1.8388312	7.66927E-06
5	739111	1373380	1.8581512	7.76449E-06
7	709894	1210211	1.7047771	7.01364E-06
11	779573	1546484	1.9837578	8.38784E-06
21	806871	1346248	1.6684798	6.83769E-06
48	789463	1426784	1.8072842	7.51418E-06
72	754937	1365892	1.8092794	7.52398E-06
96	745391	1374289	1.8437156	7.69333E-06
120	790573	1448927	1.8327555	7.63937E-06

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AN ABSTRACT OF THE THESIS OF

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Title: Reductive Dechlorination of Chlorophenols by Vitamin B₁₂.

Abstract Approved:

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The reductive dechlorination of chlorinated phenols by vitamin B₁₂, supported by the reductant Ti(III) citrate, was examined. Procedures were developed, including a novel reactor system, for conducting these experiments. Most of the experiments were conducted in either hermetically-sealed glass ampoules, which could maintain vitamin B₁₂ in the fully-reduced vitamin B_{12s} state for months, or in the novel two-chambered reactor (TCR), which could also support vitamin B_{12s} for extended periods, and could easily be sampled to perform kinetic studies.

Vitamin B_{12s} reductively dechlorinates chlorinated phenols by nucleophilic aromatic substitution of the cobalamin for a chlorine, followed by reductive cleavage of the arylcobalamin to form the reductively-dechlorinated product. Dechlorination occurs chiefly at positions *ortho* to another chlorine, but the 2 and 6 positions are extremely recalcitrant, even when another chlorine is adjacent. The proposed mechanism accounts for the observed regiospecificity, consistent with bond charge density and thermodynamic considerations.

Pentachlorophenol, all of the tetrachlorophenols, and all of the trichlorophenols were challenged with vitamin B_{12s}, and all were reductively dechlorinated to some extent. A sequential dechlorination pathway was constructed and compared to patterns exhibited by anaerobic microbial consortia. On the basis of this comparison, it was

concluded that any involvement of vitamin B₁₂ in biological reductive dechlorination of chlorinated phenols requires the intimate participation of apoenzymes to direct the regiospecificity toward the 2 and 6 chlorines on the ring.

The observed kinetics of the reductive dechlorination of pentachlorophenol by vitamin B₁₂s can be described with a first order approximation. A threefold variability in rate constants was observed. The rate of reductive dechlorination of pentachlorophenol by vitamin B₁₂s is insufficient to account for reductive dechlorination by acclimated bacteria.